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Date: March 1, 2006 **Total pages:** 75 (including cover sheet)

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Our Docket No. EU 98055 CON
Your Docket No.

Client/Matter No. 077113-00004

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MESSAGE:

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Jack L. Arbiser

Serial No.: 09/765,491 Art Unit: 1617

Filed: January 18, 2001 Examiner: J. Kim

For: *CURCUMIN AND CURCUMINOID INHIBITION OF ANGIOGENSIS*

Attached Items:

Transmittal Form PTO/SB/21; Fee Transmittal PTO/SB/17; Response and copy of Appeal Brief with references

{45084866.1}

PTO/SB/21 (09-04)
Approved for use through 07/31/2006. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	09/765,491
Filing Date	January 18, 2001
First Named Inventor	Jack L. Arbiser
Art Unit	1617
Examiner Name	Jennifer M. Kim
Attorney Docket Number	EU 98055 CON

ENCLOSURES *(Check all that apply)*

- ☑ Fee Transmittal Form
  - ☐ Fee Attached
- ☐ Amendment/Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Reply to Missing Parts/ Incomplete Application
  - ☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) _____
  - ☐ Landscape Table on CD
- ☐ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☑ Appeal Communication to TC (Appeal Notice, Brief, Reply Brief)
- ☐ Proprietary Information
- ☐ Status Letter
- ☐ Other Enclosure(s) (please identify below):

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	Pabst Patent Group LLP		
Signature			
Printed name	Patrea L. Pabst		
Date	March 1, 2006	Reg. No.	31,284

CERTIFICATE OF TRANSMISSION/MAILING

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Signature			
Typed or printed name	Chandra Russell	Date	March 1, 2006

PTO/SB/17 (01-06)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2006

☑ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 0.00

Complete if Known	
Application Number	09/765,491
Filing Date	January 18, 2001
First Named Inventor	Jack L. Arbiser
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Art Unit	1617
Attorney Docket No.	EU 98055 CON

METHOD OF PAYMENT (check all that apply)

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For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

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1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
Application Type	Fee ($)	Small Entity Fee ($)	Fee ($)	Small Entity Fee ($)	Fee ($)	Small Entity Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims _____ - 20 or HP = _____ x _____ = _____ Extra Claims / Fee ($) / Fee Paid ($)

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims _____ - 3 or HP = _____ x _____ = _____ Extra Claims / Fee ($) / Fee Paid ($)

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims: Fee ($) _____ Fee Paid ($) _____

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets _____ - 100 = _____ Extra Sheets / 50 = _____ Number of each additional 50 or fraction thereof (round up to a whole number) x _____ Fee ($) = _____ Fee Paid ($)

4. OTHER FEE(S)

Fees Paid ($)

Non-English Specification, $130 fee (no small entity discount) _____

Other (e.g., late filing surcharge): _____ _____

SUBMITTED BY

Signature		Registration No. (Attorney/Agent) 31,284	Telephone 404-879-2151
Name (Print/Type)	Patrea L. Pabst		Date March 1, 2006

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Jack L. Arbiser

Serial No.: 09/765,491 Art Unit: 1617

Filed: January 18, 2001 Examiner: Jennifer M. Kim

For: *CURCUMIN AND CURCUMINOID INHIBITION OF ANGIOGENESIS*

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE

Sir:

Responsive to the Advisory Action mailed February 21, 2006, stating that the Appeal Brief filed on November 30, 2005, is defective, enclosed is another copy of the Appeal Brief as filed on November 30, 2005. The Appeal Brief is not defective since it included both an Evidence and a Related Proceedings Appendix at the time of original filing.

Immediate action on the Appeal Brief is requested.

EU 98055 CON
077113/0004

Respectfully submitted,

Patrea L. Pabst
Reg. No. 21,284

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Applicant: Jack L. Arbiser

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Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

This is an appeal from the rejection of claims 4-6, 10-12, and 17-19 in the Office Action mailed March 31, 2005, in the above-identified patent application. A Notice of Appeal was filed on August 30, 2005 along with a two month extension of time. **A RESPONSE TO THE FINAL REJECTION, WHICH INCLUDED A NUMBER OF THE REFERENCES ATTACHED IN THE APPENDIX AS EVIDENCE, WAS FILED OCTOBER 28, 2005. IT DOES NOT APPEAR THIS RESPONSE HAS BEEN CONSIDERED BY THE EXAMINER.**

The Commissioner is hereby authorized to charge $250.00, the fee for the filing of this Appeal Brief for a small entity, to Deposit Account No. 50-3129. It is believed that no

additional fee is required with this submission. However, should an additional fee be required, the Commissioner is hereby authorized to charge the fee to Deposit Account No. 50-3129.

(1) REAL PARTY IN INTEREST

The real party in interest of this application is the assignee, Emory University, a corporation of Georgia, and the licensee, Curry Pharmaceuticals, a corporation of North Carolina.

(2) RELATED APPEALS AND INTERFERENCES

There is no pending related appeal or interference. The parent application, U.S.S.N. 09/345,712, was appealed, but the examiner allowed the application after filing of the Appeal Brief, and the application issued as U.S. patent No. 6,673,843.

(3) STATUS OF CLAIMS

Claims 4-6, 10-12, and 17-19 are pending, rejected and on appeal. Claims 1-3, 7-9, and 13-16 have been cancelled. The text of each claim on appeal, as pending, is set forth in an Appendix to this Appeal Brief.

(4) STATUS OF AMENDMENTS

The claims were last amended in an Amendment and Response filed October 1, 2004.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

Claim 4 defines a method for inhibiting symptoms associated with angiogenesis in the treatment of lymphangiogenesis, Sturge-Weber syndrome, verruca vulgaris, tuberous sclerosis, venous ulcers, molluscum contagious, seborrheic keratosis, or actinic keratosis comprising administering a collagenase inhibitor, angiogenic fumagillin derivative, 2,5-

diaryltetrahydrofuran, aminophenylphosphonic acid compound, 3-substituted oxindole derivative, thalidomide, penicillamine or IL12 in an amount effective to inhibit angiogenesis; claim 4 defines the method wherein the angiogenesis inhibitor is applied topically; and claim 6 defines the method wherein the angiogenesis inhibitor is TNP-470. The disorders to be treated are referenced at page 5, lines 15-27; the angiogenesis inhibitors are described at page 6, line 3 to page 7, line 12. Topical administration is described at page 13, lines 9-25.

Claim 10 defines a method to treat the symptoms associated with elevated basic fibroblast growth factor in angiosarcoma, hemangioendothelioma, basal cell carcinoma, squamous cell carcinoma, malignant melanoma, Kaposi's sarcoma, psoriasis, or recessive dystrophic epidermolysis bullosa, comprising administering an effective amount of a pharmaceutical composition comprising a curcuminoid in an ointment for topical administration containing between one-half percent (0.5%) and five percent (5%) of the curcuminoid or a polymer formulation for implantation. Claim 11 defines the method wherein the curcuminoid is curcumin; claim 12 defines the method wherein the curcuminoid is demethoxycurcumin. The disorders to be treated are referenced at page 5, lines 15-27; the curcuminoids are described at page 8, lines 8-24. The ointment is detailed at page 14, lines 19-21.

Claim 17 defines a method for inhibiting skin disorders: lymphangiogenesis, Sturge-Weber syndrome, verruca vulgaris, tuberous sclerosis, venous ulcers, rosacea, eczema, molluscum contagious, seborrheic keratosis, or actinic keratosis comprising administering an angiogenesis inhibitor in an amount effective to inhibit angiogenesis, wherein the angiogenesis inhibitor is either a tetracycline inhibiting collagenase, or a sulfated polysaccharide which

inhibits angiogenesis. Claim 18 defines the disorder as malignant melanoma; claim 19 defines the disorder as recessive dystrophic epidermolysis bullosa. The disorders to be treated are referenced at page 5, lines 15-27; the angiogenesis inhibitors are described at page 6, line 3 to page 7, line 12.

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The issues presented on appeal are:

(1) whether claims 4-6 and 17-19 are enabled as required by 35 U.S.C. § 112, first paragraph.

(2) whether claims 4-6 and 17 are definite as required by 35 U.S.C. § 112, second paragraph.

(3) whether claim 17 is novel under 35 U.S.C. § 102(e) over U.S. Patent No. 6,218,368 to Wirostko ("Wirostko").

(4) whether claims 4 and 5 are non-obvious under 35 U.S.C. § 103 (a) in view of U.S. Patent No. 5,190,918 in view of U.S. Patent No. 6,482,801 to Brem, et al.

(5) whether claims 4 and 5 are non-obvious under 35 U.S.C. § 103 (a) in view of U.S. Patent No. 5,190,918 to Deutch, et al., in view of U.S. Patent No. 5,654,312 to Andrulis, Jr., et al.

(6) whether claims 4 and 6 are non-obvious under 35 U.S.C. § 103 (a) in view of U.S. Patent No. 5,190,918 to Deutch, et al., in view of U.S. Patent No. 5,776,898 to Teicher, et al.

(7) whether claims 10-12 and 18 are non-obvious under 35 U.S.C. § 103 (a) in view of WO 95/18606 by Aggarwal.

(8) whether claims 10-12 and 19 are non-obvious under 35 U.S.C. § 103 (a) in view of Arbiser, et al., J. Amer. Acad. Dermatol. 40(6):925-929 (June 1999) in view of Thaloor, et al. Cell Growth & Differentiation 9, 305-312 (1998) in combination with WO 95/18606 by Aggarwal.

(7) **ARGUMENT**

a) Rejection Under 35 U.S.C. § 112, first paragraph

Claims 4-6 and 17-19 were rejected as not enabled as required by 35 U.S.C. § 112, first paragraph, for use of the phrase "an effective amount". Claims 4-6 and 17 were rejected as indefinite under 35 U.S.C. § 112, second paragraph.

(i) Enablement

The Legal Standard

The Court of Appeals for the Federal Circuit (CAFC) has described the legal standard for enablement under § 112, first paragraph, as whether one skilled in the art could make and use the claimed invention from the disclosures in the patent coupled with information known in the art, without undue experimentation (*See, e.g., Amgen v. Hoechst Marion Roussell* 314 F.3d 1313 (Fed. Cir. 2003; *Genentech, Inc. v. Novo Nordisk A/S,* 108 F3d at 165, 42 USPQ2d at 1004 (Fed. Cir. 1997) (quoting *In re Wright*, 999 F.2d 1557, 1561, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993); See also *In re Fisher*, 427 F.2d at 839, 166 USPQ at 24; *United States v. Telectronics, Inc.,* 857 F.2d 778 (Fed. Cir. 1988); *In re Stephens,* 529 F.2d 1343 (CCPA 1976)). The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation (*M.I.T. v. A.B. Fortia*, 774 F.2d 1104 (Fed. Cir. 1985)). In addition, as

affirmed by the Court in *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524 (Fed. Cir. 1987), a patent need not teach, and preferably omits, what is well known in the art.

Whether the disclosure is enabling is a legal conclusion based upon several underlying factual inquiries. See *In re Wands*, 858 F.2d 731, 735, 736-737, 8 USPQ2d 1400, 1402, 1404 (Fed. Cir.1988). As set forth in *Wands*, the factors to be considered in determining whether a claimed invention is enabled throughout its scope without undue experimentation include the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claims. In cases that involve unpredictable factors, "the scope of the enablement obviously varies inversely with the degree of unpredictability of the factors involved." *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970).

Claims 4-6 and 17-19 are enabled

A proper analysis of the *Wands* factors shows that claims 4-6 and 17-19 satisfy the enablement requirement. Applicants do not understand how the Examiner can allege that the claims are obvious, and then suggest that they are not enabled! The quantity of experimentation necessary to obtain and use the claimed angiogenesis inhibitors for the treatment of the recited disorders is **not undue.**

The angiogenesis inhibitors that may be used in the claimed methods are disclosed in the specification on page 6, line 4 to page 7, line 10 along with a number of references which thoroughly describe these agents and **effective amounts for use in humans, and methods of**

administration. In addition, the specification also teaches how to make pharmaceutical compositions of the compounds and methods that can be used to administer the drugs to patients (see, for example, pages 12-14). Furthermore, the specification lists the diseases that may be treated on page 5, line 15 to page 6, line 2. These diseases are also well-known and characterized and can be found in any medical textbook.

The fact that some experimentation is necessary does not preclude enablement; what is required is that the amount of experimentation 'must not be unduly extensive.' *In re Atlas Powder Co., v. E.I. DuPont De Nemours & Co.* , 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir.1984). There is no requirement for examples nor is there any need for examples in this application. The claimed agents are known (although **not** for the treatment of the recited disorders), well-characterized and commercially available. Therefore, one of ordinary skill in the art could routinely arrive at an effective amount of the drugs and method of delivery to treat the claimed disorders. Furthermore, as discussed below, the term "effective amount" is a common and generally acceptable term for pharmaceutical claims and is not ambiguous or indefinite, provided that a person of ordinary skill in the art could determine the specific amounts without undue experimentation, as is the case here.

Additional evidence that one can determine an effective amount of drug, even one of much less than ordinary skill in the art such as the undersigned, is provided in the accompanying abstracts of the papers by LoTempio, et al., Clin. Cancer Res. 1:11(19 Pt 1):6994-7002 (2005) (curcumin topical paste) and Li, et al., Cancer 104(6):1322-1331 (2005) (liposomally encapsulated curcumin).

In summary, it is clear from the guidance in the specification, the state of the prior art, and the level of skill in the art that one of ordinary skill in the art would be able to use the claimed angiogenesis inhibitors to treat the recited disorders without undue experimentation.

(ii) Definiteness

The Legal Standard

According to 37 CFR 1.75 (c), "One or more claims may be presented in dependent form, referring back to and further limiting another claim or claims in the same application. [...] Claims in dependent form shall be construed to include all the limitations of the claim incorporated by reference into the dependent claim." Thus if a claim depends from another claim, the dependent claim includes all the limitations of the claim from which it depends, without restating those limitations.

The test for definiteness under 35 U.S.C. § 112, second paragraph, is whether "those skilled in the art would understand what is claimed when the claim is read in light of the specification." *Orthokinetics, Inc. v. Safety Travel Chairs, Inc.*, 806 F.2d 1565, 1576, 1 USPQ2d 1081, 1088 (Fed. Cir. 1986). The fact that other language may be used in a claim is not a valid basis for a rejection under 35 U.S.C. § 112, second paragraph. The M.P.E.P. explains that the examiner's focus during examination of claims for compliance with the definiteness requirement "is whether the claim meets the threshold requirements of clarity and precision, *not whether more suitable language or modes of expression are available.*" (M.P.E.P. 2173.02, emphasis added) The M.P.E.P. further explains that "[s]ome latitude in the manner of expression and the

aptness of terms should be permitted even though the claim language is not as precise as the examiner might desire." (*Id.*)

The rejection of the claims over the term "effective amount" is legally improper. This term is a common and generally acceptable term for pharmaceutical claims and is not ambiguous or indefinite, provided that a person of ordinary skill in the art could determine the specific amounts without undue experimentation. (See, e.g., *Geneva Pharmaceuticals, Inc. v. GlaxoSmithKline PLC*, 349 F.3d 1373 (Fed. Cir. 2003); *In re Halleck*, 57 C.C.P.A. 954, 422 F.2d 911, 914 (CCPA 1970). An effective amount of the angiogenesis inhibitor is an amount as required to alleviate the symptoms of the particular disorder being treated (page 14, lines 28-29). Since the claimed angiogenesis inhibitors are known and characterized compounds (although not for the treatment of the recited disorders), one of ordinary skill in the art could arrive at an "effective amount" of any one of these drugs to treat the listed disorders.

b) Rejections over the Prior Art

(i) Rejection Under 35 U.S.C. § 102

Claim 17 was rejected under 35 U.S.C. § 102(e) as anticipated by U.S. Patent No. 6,218,368 to Wirostko ("Wirostko").

Claim 17 defines a method for inhibiting skin disorders selected from the group consisting of lymphangiogenesis, Sturge-Weber syndrome, verruca vulgaris, tuberous sclerosis, venous ulcers, rosacea, eczema, molluscum contagious, seborrheic keratosis, and actinic keratosis comprising administering to the individual in need of treatment thereof of tetracyclines inhibiting collagenase, or a sulfated polysaccharide which inhibits angiogenesis.

The Legal Standard

For a rejection of claims to be properly founded under 35 U.S.C. § 102, it must be established that a prior art reference discloses each and every element of the claims. *Hybritech Inc v Monoclonal Antibodies Inc*, 231 USPQ 81 (Fed. Cir. 1986), *cert. denied*, 480 US 947 (1987); *Scripps Clinic & Research Found v Genentech Inc*, 18 USPQ2d 1001 (Fed. Cir. 1991). The Federal Circuit held in *Scripps*, 18 USPQ2d at 1010:

> Invalidity for anticipation requires that all of the elements and limitations of the claim are found within a single prior art reference. . . *There must be no difference* between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention. (Emphasis added)

A reference that fails to disclose even one limitation will not be found to anticipate, even if the missing limitation could be discoverable through further experimentation. As the Federal Circuit held in *Scripps, Id.*:

> [A] finding of anticipation requires that all aspects of the claimed invention were already described in a single reference: a finding that is not supportable if it is necessary to prove facts beyond those disclosed in the reference in order to meet the claim limitations. The role of extrinsic evidence is to educate the decision-maker to what the reference meant to persons of ordinary skill in the field of the invention, not to fill in the gaps in the reference.

For a prior art reference to anticipate a claim, it must enable a person skilled in the art to practice the invention. "A claimed invention cannot be anticipated by a prior art reference if the allegedly anticipatory disclosures cited as prior art are not enabled." Amgen, Inc. v. Hoechst Marion Roussel, Inc., 314 F.3d 1313, 1354, 65 USPQ2d 1385, 1416 (Fed. Cir. 2003). See Bristol-Myers Squibb v. Ben Venue Laboratories, Inc., 246 F.3d 1368, 1374, 58 USPQ2d 1508, 1512 (Fed. Cir. 2001) ("To anticipate the reference must also enable one of skill in the art to make and use the claimed invention.").

Factual Analysis

Wirostko

Wirostko describes systemic administration of a tetracycline to treat acne rosacea. Wirostko administers a tetracycline for its common antibiotic activity and is using the common misnomer of acne rosacea to refer to acne characterized by redness. Acne is actually an infection of the skin; rosacea is a different disease. Attached in the appendix is a printout from the National Rosacea Society that explains the criteria, causes, and differences with acne (and confusion in nomenclature), thereby establishing that Wirostko does not disclose treatment as defined by claim 17.

(ii) Rejections Under 35 U.S.C. § 103

Claims 4 and 5 were rejected under 35 U.S.C. § 103 (a) as being obvious over U.S. Patent No. 5,190,918 to Deutch, et al., in view of U.S. Patent No. 6,482,801 to Brem, et al. or Deutch, et al., in view of U.S. Patent No. 5,654,312 to Andrulis, Jr. Claims 4-6 were rejected under 35 U.S.C. 103 as obvious over Deutch in view of U.S. Patent No. 5,776,898 to Teicher, et al.

Claims 10-12 and 18 were rejected as obvious over WO 95/18606 to Aggarwal. Claims 10-12 and 19 were also rejected as obvious under 35 U.S.C. 103 over Arbiser, et al. J. Amer. Acad. Dermatol. 40, No. 6, 925-929 (June 1999) in view of Thaloor, et al. Cell Growth & Differentiation 9, 305-312 (April 1998) and Aggarwal.

The Legal Standard

"References relied upon to support a rejection under 35 USC 103 must provide an enabling disclosure, i.e., they must place the claimed invention in the possession of the public." *Application of Payne*, 606 F.2d 303, 314, 203 U.S.P.Q. 245 (C.C.P.A. 1979); *see Beckman Instruments, Inc. v. LKB Produkter AB*, 892 F.2d 1547, 13 U.S.P.Q.2d 1301 (Fed. Cir. 1989). A publication that is insufficient as a matter of law to constitute an enabling reference may still be relied upon, but only for what it discloses. *See Reading & Bates Constr. Co. v. Baker Energy Resources Corp.*, 748 F.2d 645, 651-652, 223 U.S.P.Q. 1168 (Fed. Cir. 1984); *Symbol Technologies, Inc. v. Opticon, Inc.*, 935 F.2d 1569 (Fed. Cir. 1991).

"Focusing on the obviousness of substitutions and differences, instead of on the invention as a whole, is a legally improper way to simplify the often difficult determination of obviousness." *Gillette Co. v. S.C. Johnson & Sons, Inc.*, 919 F.2d 720, 724, 16 U.S.P.Q.2d 1923 (Fed. Cir. 1990); *see Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1383, 231 U.S.P.Q. 81, 93 (Fed. Cir. 1986). "One cannot use hindsight reconstruction to pick and choose among isolated disclosures on the prior art to deprecate the claimed invention." *In re Fine*, 837 F.2d 1071, 1075 (Fed. Cir. 1988).

The prior art must provide one of ordinary skill in the art with the motivation to make the proposed modifications needed to arrive at the claimed invention. *See In re Geiger* , 815 F.2d 686, 2 U.S.P.Q.2d 1276 (Fed. Cir. 1987); *In re Lalu and Foulletier*, 747 F.2d 703, 705, 223 U.S.P.Q. 1257, 1258 (Fed. Cir. 1984). Claims for an invention are not *prima facie* obvious if the primary references do not suggest all elements of the claimed invention and the prior art does not suggest the modifications that would bring the primary references into conformity with the application claims. *In re Fritch*, 23 U.S.P.Q.2d, 1780 (Fed. Cir. 1992). *In re Laskowski*, 871 F.2d 115 (Fed. Cir. 1989). This is not possible when the claimed invention achieves more than what any or all of the prior art references allegedly suggest, expressly or by reasonable implication.

"Obviousness is determined as follows:

'a proper analysis under § 103 requires, *inter alia*, consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success.' *Noelle v. Lederman*, 355 F.3d 1343, 69 USPQ2d 1508 (Fed. Cir. 2004) "Both [a] suggestion [to make a claimed composition or process] and [a] reasonable expectation of success 'must be founded in the prior art, not in the applicant's disclosure.' " *Velander v. Garner*, 348 F.3d 1359, 68 USPQ2d 1769 (Fed. Cir. 2003) "Both the suggestion and the reasonable expectation of success 'must be founded in the prior art, not in the applicant's disclosure.' *Id.; see also In re Dow Chem. Co.*, 837 F.2d 469, 473 (Fed. Cir. 1988)."

Factual Analysis

Claims 4-6

Claims 4-6 define a method for inhibiting symptoms associated with angiogenesis in the treatment of skin disorders: lymphangiogenesis, Sturge-Weber syndrome, verruca vulgaris, tuberous sclerosis, venous ulcers, molluscum contagious, seborrheic keratosis, or actinic keratosis, comprising administering a collagenase inhibitor, angiogenic fumagillin derivative, 2,5-diaryltetrahydrofuran, aminophenylphosphonic acid compound, 3-substituted oxindole derivative, thalidomide, penicillamine or IL12 in an amount effective to inhibit angiogenesis (claim 4), wherein the angiogenesis inhibitor is applied topically (claim 5), and where the angiogenesis inhibitor is specifically TNP-470.

Deuth

Deuth, et al. allegedly shows that angiogenesis activity is the ability to enhance the formation of lymph vessels. This is completely contrary to any common definition of angiogenesis, which is defined in the application at page 2 of the application and in the literature as relating to the initiation and growth of blood vessels.

Deuth in combination with Brem

Brem, et al. acknowledges that minocycline is an angiogenesis inhibitor and a collagenase inhibitor.

There is nothing that would lead one skilled in the art of treating lymphangiogenesis to adopt the peculiar description of Deuth and combine it with Brem, et al., with any expectation of success. Attached in the Appendix as evidence is an article, Jussila and Alitalo, "Vascular

Growth Factors and Lymphangiogenesis" Physiol. Rev. 82, 673-700 (2002) that explains and differentiates angiogenesis and lymphangiogenesis. While not prior art, it establishes the important differences between angiogenesis and lymphanigiogenesis. While both are extremely important, they are not the same, and a reference to one would not lead one to assume the same with respect to the other, and certainly not that a drug effective in one condition would be effective in the other condition.

Deuth in combination with Andrulis

Deuth, et al. is discussed above. Andrulis does not lead one to extrapolate from lymphangiogenesis to angiogenesis nor that there would be any expectation that thalidomide, referenced by Andrulis with respect to inhibiting angiogenesis, would be effective in preventing lymphangiogenesis.

Claim 6

Deuth in combination with Teicher

Deuth is discussed above. Teicher is no different than Andrulis or Brem. It also discloses only a compound known to inhibit angiogenesis, TNP-470, not lymphangiogenesis. There is nothing to lead one of ordinary skill in the art to extrapolate from angiogenesis to lymphagiogenesis; no reference that would motivate one skilled in the art to have a reasonable expectation of success in using a compound known to inhibit angiogenesis to inhibit lymphangiogenesis. Therefore, although claim 6 is specific to the use of TNP-470, there is no teaching leading one of ordinary skill in the art to use this compound for the treatment of any of the specified conditions.

Claims 10-12, 18 and 19

Claims 10-12 define a method to treat the symptoms associated with elevated basic fibroblast growth factor in angiosarcoma, hemangioendothelioma, basal cell carcinoma, squamous cell carcinoma, malignant melanoma, Kaposi's sarcoma, psoriasis, or recessive dystrophic epidermolysis bullosa, comprising administering an effective amount of a pharmaceutical composition comprising a curcuminoid in an ointment for topical administration containing between one-half percent (0.5%) and five percent (5%) of the curcuminoid or a polymer formulation for implantation (claim 10); wherein the curcuminoid is curcumin (claim 11); and wherein the curcuminoid is demethoxycurcumin (claim 12). Claim 18 defines the disorder as malignant melanoma and claim 19 defines the disorder as recessive dystrophic epidermolysis bullosa.

Aggarwal

Aggarwal has been cited as making obvious claims 10-12 and 18. Claim 10 defines a method to treat the symptoms associated with elevated basic fibroblast growth factor in a disorder selected from the group consisting of angiosarcoma, hemangioendothelioma, basal cell carcinoma, squamous cell carcinoma, malignant melanoma, Kaposi's sarcoma, psoriasis, and recessive dystrophic epidermolysis bullosa, comprising administering to the individual in need of treatment an effective amount of a pharmaceutical composition comprising a curcuminoid in combination with a pharmaceutically acceptable carrier to inhibit angiogenesis, wherein the carrier is an ointment for topical administration containing between one-half percent (0.5%) and five percent (5%) of the curcuminoid or a polymer formulation for implantation.

It is difficult to understand how the use of this formulation could be obvious, since the same examiner previously allowed claims to the composition *per se*, in the now issued parent application, U.S. Patent No. 6,673,843.

Indeed, the composition is not obvious, nor is the use in any of the defined conditions obvious. The examiner has cited no evidence why one skilled in the art would have any motivation to treat completely different disorders with the claimed formulation, nor why one would have any expectation of success based on a reference using a hugely different amount of drug (1 microgram to 100 milligrams) as compared to the amount in the claimed formulation. It is not enough to make allegations that a reference makes something obvious; the rejection must be based on a factual analysis that one skilled in the art would be led to the difference defined by the claims, and have a reasonable expectation of success. The examiner has failed to provide any support for the rejection.

Arbiser in view of Thaloor and Aggarwal

Arbiser is not prior art to this application. This application claims priority to June 30, 1999. Arbiser was published in June 1999. To the extent it might be prior art to this application under 35 U.S.C. 102(a), it is clear that it could be removed as prior art as the inventor's own publication (note that it is Dr. Arbiser to whom inquiries are to be directed). However, as the examiner has noted, this paper does not describe administering curcumin, which is the subject matter of the claimed method.

Thaloor does not make up for this deficiency. Arbiser says there are at least two mechanisms – the role of basic fibroblast growth factor and angiogenesis involved in recessive

dystrophic epidermolysis bullosa. Nothing the examiner cites lead one of ordinary skill in the art to expect curcumin to treat both mechanisms, therefore there would be no expectation of success. One would instead believe that TNP-470, which is administered systemically, not curcumin, administered as an ointment, would be effective. Aggarwal, discussed above, does not make up for this deficiency.

(8) SUMMARY AND CONCLUSION

In summary, the claims are definite, novel and not obvious over the prior art.

Respectfully submitted,

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Appendix I: Claims on Appeal

Claims 1-3 (canceled)

4. (previously amended) A method for inhibiting symptoms associated with angiogenesis in the treatment of skin disorders selected from the group consisting of lymphangiogenesis, Sturge-Weber syndrome, verruca vulgaris, tuberous sclerosis, venous ulcers, molluscum contagious, seborrheic keratosis, and actinic keratosis comprising administering to the individual in need of treatment thereof an angiogenesis inhibitor wherein the angiogenesis inhibitor is selected from the group consisting of collagenase inhibitors, angiogenic fumagillin derivatives, 2,5-diaryltetrahydrofurans, aminophenylphosphonic acid compounds, 3-substituted oxindole derivatives, thalidomides, penicillamine and IL12 in an amount effective to inhibit angiogenesis.

5. (original) The method of claim 4 wherein the angiogenesis inhibitor is applied topically.

6. (previously presented) The method of claim 5 wherein the angiogenesis inhibitor is TNP-470.

Claims 7-9 (canceled)

10. (previously amended) A method to treat the symptoms associated with elevated basic fibroblast growth factor in a disorder selected from the group consisting of angiosarcoma, hemangioendothelioma, basal cell carcinoma, squamous cell carcinoma, malignant melanoma, Kaposi's sarcoma, psoriasis, and recessive dystrophic epidermolysis bullosa, comprising administering to the individual in need of treatment an effective amount of a pharmaceutical

composition comprising a curcuminoid in combination with a pharmaceutically acceptable carrier to inhibit angiogenesis, wherein the carrier is an ointment for topical administration containing between one-half percent (0.5%) and five percent (5%) of the curcuminoid or a polymer formulation for implantation.

11. (previously presented) The method of claim 10 wherein the curcuminoid is curcumin.

12. (previously presented) The method of claim 10 wherein the curcuminoid is demethoxycurcumin.

Claims 13-16 (canceled)

17. (previously presented) A method for inhibiting skin disorders selected from the group consisting of lymphangiogenesis, Sturge-Weber syndrome, verruca vulgaris, tuberous sclerosis, venous ulcers, rosacea, eczema, molluscum contagious, seborrheic keratosis, and actinic keratosis comprising administering to the individual in need of treatment thereof an angiogenesis inhibitor in an amount effective to inhibit angiogenesis, wherein the angiogenesis inhibitor is selected from the group consisting of

tetracyclines inhibiting collagenase, and

a sulfated polysaccharide which inhibits angiogenesis.

18. (previously presented) The method of claim 10 wherein the disorder is malignant melanoma.

19. (previously presented) The method of claim 10 wherein the disorder is recessive dystrophic epidermolysis bullosa.

Appendix II: Evidence on Appeal

LoTempio, et al., Clin. Cancer Res. 1:11(19 Pt 1):6994-7002 (2005) (curcumin topical paste)

Li, et al., Cancer 104(6):1322-1331 (2005) (liposomally encapsulated curcumin).

Printout from the National Rosacea Society

Jussila and Alitalo, "Vascular Growth Factors and Lymphangiogenesis" Physiol. Rev. 82, 673-700 (2002)

Abstract for Arbiser, et al. J. Am. Acad. Dermatol. 40(6 Pt 1):925-929 (June 1999)

1: J Am Acad Dermatol. 1999 Jun;40(6 Pt 1):925-9. Related Articles, Links

J Am Acad Dermatol

The antiangiogenic agents TNP-470 and 2-methoxyestradiol inhibit the growth of angiosarcoma in mice.

Arbiser JL, Panigrathy D, Klauber N, Rupnick M, Flynn E, Udagawa T, D'Amato RJ.

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BACKGROUND: Endothelial malignancies, such as angiosarcoma and hemangioendothelioma, are often resistant to chemotherapy and surgery, and may result in death. Improved means of therapy are needed for these disorders. OBJECTIVE: We wanted to determine whether angiosarcoma can be treated with angiogenesis inhibitors in mice. METHODS: Mice were inoculated with a cell line that gives rise to angiosarcoma and were treated with the angiogenesis inhibitors 2-methoxyestradiol and TNP-470. Response to therapy was monitored by measurement of tumors. RESULTS: TNP-470 caused an 84% reduction in tumor size, and 2-methoxyestradiol caused a 68% reduction in tumor size. CONCLUSION: Angiogenesis inhibitors are highly effective in treatment of angiosarcoma in mice. Clinical trials of these agents in humans with angiosarcoma and hemangioendothelioma are warranted.

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Curcumin Suppresses Growth of Head and Neck Squamous Cell Carcinoma

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Abstract **Purpose:** The purpose of this study was to determine whether curcumin would trigger cell death in the head and neck squamous cell carcinoma (HNSCC) cell lines CCL 23, CAL 27, and UM-SCC1 in a dose-dependent fashion.

Experimental Design: HNSCC cells were treated with curcumin and assayed for *in vitro* growth suppression using 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide and fluorescence-activated cell sorting analyses. Expression of p16, cyclin D1, phospho-IκB, and nuclear factor-κB (NF-κB) were measured by Western blotting, gel shift, and immunofluorescence.

Results: Addition of curcumin resulted in a dose-dependent growth inhibition of all three cell lines. Curcumin treatment resulted in reduced nuclear expression of NF-κB. This effect on NF-κB was further reflected in the decreased expression of phospho-IκB-α. Whereas the expression of cyclin D1, an NF-κB–activated protein, was also reduced, there was no difference in the expression of p16 at the initial times after curcumin treatment. *In vivo* growth studies were done using nude mice xenograft tumors. Curcumin was applied as a noninvasive topical paste to the tumors and inhibition of tumor growth was observed in xenografts from the CAL 27 cell line.

Conclusions: Curcumin treatment resulted in suppression of HNSCC growth both *in vitro* and *in vivo*. Our data support further investigation into the potential use for curcumin as an adjuvant or chemopreventive agent in head and neck cancer.

Head and neck squamous cell carcinoma (HNSCC) represents 5% of all cancers diagnosed annually in the United States (1). Current treatment protocols for advanced head and neck cancer often entail a disfiguring and risky surgical operation. In addition, radiation therapy, chemotherapy, and surgery result in tremendous morbidity for patients with HNSCC. Despite the best efforts of previous research, survival rates for late-stage HNSCC remain dismal, and it is apparent that a different approach to treatment is needed. As such, investigations of potential alternative therapies for HNSCC with fewer associated toxicities are continuing.

Curcumin, commonly known as the spice turmeric, is derived from the rhizome of the East Indian plant *Curcuma longa* (2, 3). This spice is used as a flavoring and coloring agent, as a food preservative, and also has been used in Ayurvedic medicine for over 6,000 years. Curcumin is soluble only in organic solvents such as ethanol or DMSO. Crude curcumin has a natural yellow hue and its components include curcumin, demethoxycurcumin, and bisdemethoxycurcumin, commonly called curcuminoids. Curcumin has been shown to suppress cellular proliferation in breast, colon, oral, and other cancers and is currently being studied for its chemopreventive and anticancer properties (4–10).

Epidemiologic studies have attributed the low incidence of colon cancers in India to the chemopreventive and antioxidant properties of diets high in starch and curcumin (11). Curcumin has also been found to have anti-inflammatory properties (12). It increases the level of glutathione-*S*-transferase and, thus, up-regulates the synthesis of glutathione, an antioxidant (13, 14). Other beneficial effects ascribed to curcumin are its wound-healing, antiviral, anti-infectious, and antiamyloidogenic properties, suggesting its use for treatment of Alzheimer's disease (15).

Nuclear factor-κB (NF-κB) is an inducible transcription factor that is involved in the activation of a number of cell processes, including cell growth and apoptosis (16). This factor is activated in many cell types in response to a broad range of stimuli, which include mitogens, inflammatory cytokines such as interleukin-1 and tumor necrosis factor (TNF), and extracellular stress such as UV light and cigarette smoke. Thus, NF-κB is a key cell cycle regulator whose activity results in enhanced transcription of growth-stimulating genes such as *cyclin D1*, *COX2*, and *Bcl-2*.

One mechanism that may play a role in the anticancer properties of curcumin may be related to the down-regulation of

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Note: M.S. Veena and H.L. Steele contributed equally to this work.

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NF-κB (17). Our hypothesis is that curcumin inhibits the activity of NF-κB and suppresses growth of HNSCC *in vitro* and *in vivo*. Although many studies have been done on the actions of curcumin *in vitro*, including a recent study of HNSCC cell lines (9), few studies of human cancer xenografts in nude mice have been done and no studies of the effect of curcumin on HNSCC *in vivo* have been done. In the present investigation, we studied the effect of curcumin on HNSCC *in vitro* and *in vivo* using a nude mouse xenograft model and found suppression of cell growth. In the nude mice studies, we compared two different methods of delivery of curcumin: intratumoral injection and topical application of the curcumin paste onto the tumor. To our knowledge, we are the first to attempt *in vivo* studies using curcumin paste in nude mice. Use of this method of delivery could overcome the problem of low bioavailability encountered in studies of oral curcumin in colorectal and other cancers in mice and in humans (10, 18).

Materials and Methods

Cell lines. The HNSCC cell lines CCL23, CAL27, UM-SCC1, and UM-SCC14A were used, which represent laryngeal, tongue, and oral cavity carcinomas. CCL23 and CAL27 were obtained from the American Type Culture Collection (Manassas, VA), and the UM-SCC1 and UM-SCC14A lines were obtained from Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI). CCL23 cells were grown in Eagle's MEM (Life Technologies, Grand Island, NY) containing 1 mmol/L glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin, 0.5 μg/mL fungizone, and 10% FCS (Invitrogen; Carlsbad, CA). The other cell lines were grown in DMEM containing high glucose (4,500 μg/mL) and 1 mmol/L glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin, 0.5 μg/mL fungizone, and 10% FCS. Cells were grown in serum-free media for 24 hours before treatment with curcumin (Sigma, St. Louis, MO).

Curcumin treatment of head and neck squamous cell carcinoma cell lines. Cell lines were plated in 96-well plates, with 10,000 cells per well, and allowed to grow for 24 hours. The cells were then serum starved for 24 hours to synchronize cells in G_0 phase of cell cycle. Curcumin (purity 65%; Sigma) was dissolved in the organic solvent DMSO. The stock solution of curcumin is 100 mmol/L in DMSO. This was diluted for final concentrations ranging from 50 to 400 μmol/L. Final DMSO concentrations ranged from 0.05% for 50 μmol/L curcumin to 0.4% for 400 μmol/L curcumin. This treatment was administered for 8 hours, which is also the half-life of curcumin *in vitro* (9). These doses were chosen because treatment with 25 μmol/L curcumin for 8 hours resulted in minimal effect on HNSCC cells, whereas treatment with 400 μmol/L curcumin resulted in nearly 100% cell death. Control wells were treated with DMSO in amounts equal to the concentration of DMSO necessary to solubilize curcumin. Cells were then allowed to incubate in serum-containing media at 37°C for an additional 8 hours and cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay system.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay for cell viability. Briefly, growth media were aspirated out, taking care not to disturb the residual cell mass at the bottom of the plate. Following this, 0.5 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (1 mg/mL in complete medium; Sigma) was added to each well, followed by incubation at 37°C for 1 hour until the solution turned purple. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was aspirated out of the wells and air-dried for 5 minutes. Serial dilution of the cells was done in isopropanol, and absorbance values were read in an ELISA plate reader at 570 nm.

Western blot analysis. The cell monolayer (10^6 cells in a 100 mm tissue culture dish) was rapidly rinsed twice with ice-cold PBS and lysed in 1 mL of ice-cold lysis buffer. The lysis buffer contained 0.1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L EDTA, 25 mmol/L β-glycerophosphate, 0.1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1 μg/mL of leupeptin and aprotinin, 0.2% Triton X-100, and 0.3% NP40 in 50 mmol/L Tris-HCl/150 mmol/L NaCl (pH 7.5). The lysates were centrifuged at 12,000 × *g* at 4°C for 10 minutes and the supernatants were collected. Aliquots of supernatants containing 20 μg of protein and prestained protein markers were subjected to SDS-PAGE in 10% gels under reducing conditions and proteins were electrotransferred to polyvinylidene difluoride membranes (Millipore, Inc., Bedford, MA). After blocking nonspecific binding by incubation with 5% nonfat milk in PBS, the membranes were incubated with polyclonal antibodies (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) using the established protocol (17).

Gel shift assay. Single-stranded oligonucleotides representing the binding site of NF-κβ (shown as underlined sequence in forward oligonucleotide, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, and reverse oligonucleotide, 5′-GCCTGGGAAAGTCCCCTCAACT-3′) were synthesized and annealed to form the double-stranded DNA using standard protocols. The double-stranded DNA was labeled at the 5′ end using γ-^{32}P-labeled ATP in the presence of T4 polynucleotide kinase. The labeled DNA was separated from the free label and mixed with the cell line extract. The incubation was done at room temperature for 30 minutes and the mixture was loaded onto a 6% PAGE gel. Electrophoresis was carried out in 0.5% Tris-borate EDTA buffer at 50 V for 2 hours until the loading dye was visualized near the bottom of the gel. The gel was dried in vacuum and exposed to X-ray films for 1 to 3 days. The mobility of the NF-κβ–bound oligonucleotide will be retarded compared with the unbound oligonucleotide. Thus, the bound radioactivity will be retained at the NF-κβ protein site. Unbound oligonucleotide will be at the bottom of the gel. An NF-κβ oligonucleotide sequence containing a mutation at the binding site and oligonucleotide sequences representing the activator protein 1 binding site (Santa Cruz Biotechnologies) were used as controls.

Immunofluorescence. The cell lines were grown to 50% to 60% confluence on coverslips and then fixed after various treatments in 3.5% paraformaldehyde at 4°C for 5 hours and then washed (thrice for 5 minutes each) with PBS, also at 4°C. Immediately, cells were treated with freshly prepared 0.25% ammonium chloride for 5 minutes and washed thrice with PBS for 5 minutes each. Cells were then permeabilized with 0.2% Triton X-100 for 10 minutes followed by blocking with 1% bovine serum albumin in PBS for 15 minutes. Next, cells were treated with either monoclonal antibody for NF-κB (5 μg/mL; Santa Cruz Biotechnologies) or with monoclonal antibody for cyclin D1 (5 μg/mL; Calbiochem, San Diego, CA) in 1% BSA for 30 minutes followed by treatment with Alexa-568 anti-mouse monoclonal antibody (1:400; Molecular Probes, Eugene, OR) for 30 minutes. Cells were then washed and mounted on a microscope slide with Vectashield (Vector Laboratories, Burlingame, CA). Fluorescence imaging was done using a Leica DMIRB digital microscope equipped with an ORCA CCD camera. Images were recorded using Open Lab 3.1 (Improvision, Inc., Boston, MA) software and analyzed using the same software or using Adobe Photoshop 7.1.

Fluorescence-activated cell sorting analysis. The curcumin-treated and untreated cell lines were analyzed using a Becton Dickinson FACScan Analytic Flow Cytometer (Becton Dickinson, San Jose, CA). Briefly, 1 × 10^6 cells were suspended in ice-cold hypotonic DNA staining solution containing propidium iodide. Samples were protected from light and analyzed within 1 hour with the flow cytometer.

Annexin V-FITC assay for apoptosis. The apoptosis assay was carried out with the Annexin V-FITC kit using the protocol of the manufacturer (Oncogene Research Products, Boston, MA). Briefly, 5 × 10^5 cells in a 0.5 mL suspension of the culture medium were centrifuged at 1,000 × *g* for 5 minutes, suspended in 0.5 mL of cold PBS, centrifuged again at the low speed for 5 minutes, and resuspended in 0.5 mL of cold Annexin V binding buffer [10 mmol/L HEPES, (pH 7.4), 150 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 4% BSA]. Annexin V-FITC (1.25 μL) was added and incubated in the dark at room temperature

for 15 minutes. The treated cells were centrifuged at the low speed for 5 minutes and suspended again in 0.5 mL of cold binding buffer. Propidium iodide (10 μL) was added and the cells were analyzed on a Becton Dickinson FACScan Analytic Flow Cytometer. Annexin V-FITC fluorescence (FL1) was detected at 518 nm and the propidium iodide (FL2) at 620 nm.

Head and neck squamous cell carcinoma xenograft tumors in mice. Five-week-old female athymic nude mice (*nu/nu*; Harlan, Chicago, IL) were utilized for *in vivo* experiments. Animals were housed in sterile rodent microisolator caging, with filtered cage top. Three to four animals were housed in each cage, in caging in which the animals rested directly on bedding. They were given free access to sterile water and food. All cages, covers, and bedding were sterilized weekly. All animal procedures were approved by the Institutional Animal Care and Use Committee of the West Los Angeles Veterans Affairs Medical Center, in accordance with the USPHS Policy on Humane Care and Use of Laboratory Animals. Animals were injected with 1 million cells of the HNSCC cell line in the left or right flank to form xenograft tumors.

In vivo *intratumoral injection of curcumin.* Xenograft tumors were grown for 14 days to a volume of 27 mm^3 and were injected daily for 5 days with 0.1 mL of either DMSO control or the experimental solution of curcumin/DMSO ranging in concentration from 50 to 250 μmol/L, increased incrementally over 5 weeks. These concentrations were chosen because of the *in vitro* results. Higher concentrations will not differentiate the effect of curcumin from DMSO because high concentrations of DMSO could also result in cell death. Because we did not observe any tumor suppression after each week of treatment with the dose of curcumin used, the dose was increased every week. Therefore, during the first week, tumors were injected with 50 μmol/L curcumin daily. During the second week, tumors were injected with 100 μmol/L curcumin daily. During the third week, tumors were injected with 150 μmol/L curcumin daily. During the fourth week, tumors were injected with 200 μmol/L curcumin daily. During the fifth week, tumors were injected with 250 μmol/L curcumin daily. Final DMSO concentrations ranged from 0.05% for the 50 μmol/L curcumin dose to 0.25% for the 250 μmol/L curcumin dose. Control tumors were injected with 0.1 mL of DMSO daily for 5 weeks. Tumor size was measured weekly. At the end of 7 weeks, animals were euthanized, blood samples were taken, and organs were harvested for toxicology.

Topical paste application of curcumin. Tumors were grown for 14 days, as described before, to a volume of 27 mm^3 and then a topical paste of curcumin in saline was applied daily onto the tumor. Curcumin is insoluble in saline; therefore, a thick paste was formed with 1 g of curcumin added to 1 mL of saline. The entire tumor was covered by the paste and new paste was applied to the tumors daily. Topical saline or topical DMSO alone was applied daily as control treatment. Tumor size was measured weekly for 3 weeks. At the end of 3 weeks, animals were euthanized, blood samples were taken, and organs were harvested for toxicology.

Toxicology studies. Blood samples were taken from the mice before euthanasia Complete blood counts, as well as levels of electrolytes, urea nitrogen, creatinine, and sorbitol dehydrogenase, a mouse liver function enzyme, were measured to determine effects on bone marrow, kidney, and liver function. Mouse organs, including skeletal muscle, thyroid, salivary gland, pancreas, brain, liver, and gastrointestinal organs, were also studied by a pathologist to determine inflammatory, hyperplastic, or neoplastic changes that could be due to curcumin toxicity.

Statistical analysis. The data were analyzed by both ANOVA and two-group *t* tests. Each ANOVA model used three terms: group (curcumin versus DMSO alone), dose, and the group by dose interaction effect. Next, the two-group *t* test was used to compare the two groups at each of the doses in the two experiments.

Results

Growth inhibition of head and neck squamous cell carcinoma cell lines with curcumin. Four different cell lines were tested for growth inhibition with varying concentrations of curcumin. CCL23 represented a slow-growing laryngeal tumor, whereas CAL27, UM-SCC1, and UM-SCC14A represented more aggressive oropharyngeal and oral cavity cancers. DMSO, which was used for dissolving curcumin, served as the control. For each cell line, at least three independent experiments were carried out and each was done in triplicate in 96-well plates.

Treatment of CCL23 with curcumin in increasing doses resulted in dose-dependent cytotoxicity (Fig. 1A). Similarly, treatment of CAL27 and UMSCC-1 cell lines with curcumin also resulted in cell death (Fig. 1B and C). There was dose-dependent cell death in three cell lines, with maximal killing at the highest concentrations (150 μmol/L for CCL23 and SCC-1, and 300 μmol/L for CAL27). Curcumin treatment of SCC-14A resulted in growth inhibition, similar to CAL27 (data not shown). ANOVA analysis indicated that for the individual

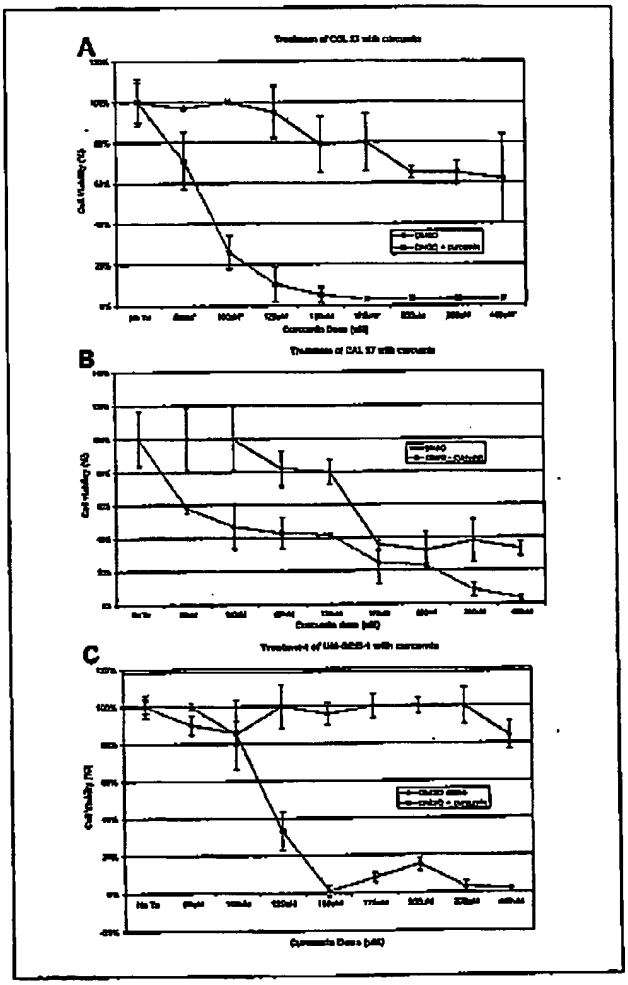

Fig. 1. Growth inhibition of HNSCC cells *in vitro* with curcumin. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were done on CCL23 (*A*), CAL27 (*B*), and UM-SCC1 (*C*) cells following treatment with increasing doses of curcumin dissolved in DMSO. DMSO alone has some inhibitory effect; however, curcumin has a significantly greater cytotoxic effect in all three cell lines ($P < 0.0001$). There is near-complete cell death with 150 μmol/L of curcumin in CCL23 and UM-SCC1 cell lines, and a concentration of 400 μmol/L is required to achieve complete cell killing in CAL27 cells.

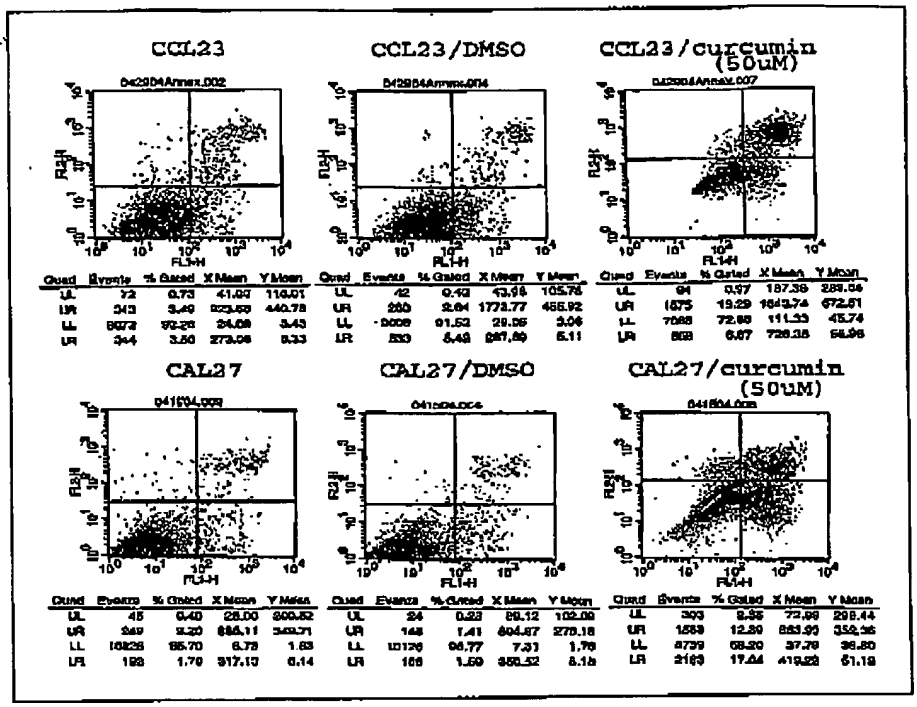

Fig. 2. Fluorescence-activated cell sorting analysis of HNSCC cells treated with curcumin. *Bottom left*, representing live cells, shows decreased survival by treatment with curcumin in both CCL23 and CAL27 cell lines. There is a higher proportion of early apoptotic cells (*bottom right*) in CAL27 cells.

cell lines and for the treatment group (curcumin versus DMSO), the correlation between cell death and increasing concentrations of curcumin was significant ($P < 0.0001$).

Fluorescence-activated cell sorting analysis was done on cells treated with 50 μmol/L of curcumin, the concentration inducing measurable cell death in all of the cell lines. As seen with the viability assay, compared with the control DMSO-treated cells, both CCL23 and CAL27 cell lines showed increased cell death with the addition of curcumin (Fig. 2). CCL23 cells had a marginally lower percentage of total cell death (27% compared with 33% in CAL27) and a lower percentage of cells in early apoptosis (6% compared with 17% in CAL 27 cells).

Reduced nuclear expression of nuclear factor-κβ in CCL23 and CAL27 cell lines. To determine the mechanism of the growth suppressive effect of curcumin, expression of cell cycle and apoptotic genes was measured. Protein extracts prepared from cells treated with 50 μmol/L curcumin for different time periods (from 2 to 6 hours) were analyzed by the gel shift assay to determine the level of NF-κβ. Extract was also prepared from untreated cells and cells treated with 0.05% DMSO, the amount present in curcumin preparations, as controls. The analysis showed a reduction in the level of NF-κβ within 4 hours of curcumin treatment (Fig. 3A). There was a dramatic decrease in NF-κβ after 6 hours, correlating with increased cell death in these cell lines. Specificity of NF-κβ binding was observed by the loss of binding with the inclusion of cold double-stranded NF-κβ oligonucleotide, but not with the addition of a mutant double-stranded NF-κβ oligonucleotide or a nonspecific double-stranded activator protein 1 oligonucleotide.

To confirm that curcumin treatment indeed resulted in reduced expression of nuclear NF-κβ, immunofluorescence was done using NF-κβ–specific antibody. Cells were also treated with TNF-β to stimulate nuclear transport of NF-κβ. There was increased nuclear expression of NF-κβ with the addition of TNF-β for 1 hour to the CCL23 cells (Fig. 3B). This expression was marginally altered by a 30-minute posttreatment with curcumin. However, a 30-minute pretreatment with curcumin before the addition of TNF-β showed a significant reduction in NF-κβ expression, clearly indicating the effect of curcumin on the expression of nuclear NF-κβ, thereby resulting in growth inhibition.

Reduced phosphorylation of Iκβ (phospho-IkB-α-Ser^{32}) and decreased expression of cyclin D1 in curcumin-treated cell lines. Whereas CCL23 cells express p16 at a measurable level, p16 expression is very low or absent in CAL27 cells. As compared with CCL23 cells, CAL 27 cells have higher level expression of cyclin D1. Thus, the level of p16 expression and Iκβ was determined in CCL23 cells and that of cyclin D1 and Iκβ in CAL27 cells (Fig. 4). Treatment with 25 μmol/L of curcumin for 8 hours did not alter the expression of p16 and Iκβ in CCL23 cells, or the expression of cyclin D1 in CAL27 (Fig. 4A). The expression of Iκβ was not altered in CAL27 cells following treatment with 25 μmol/L of curcumin (data not shown). However, there was a significant difference in their expression with the addition of 50 μmol/L curcumin. The p16 level in CCL23 was reduced 6 hours posttreatment, possibly reflecting enhanced cell death by this time period (Fig. 4B). The decreased expression of cyclin D1 and Iκβ in CAL27 was observed within 2 hours following treatment with curcumin. Complete absence of expression was observed after 4 hours of curcumin treatment for cyclin D1 and after 6 hours for Iκβ.

To confirm that cyclin D1 expression was reduced within 2 hours of curcumin treatment, the more sensitive immunofluorescence strategy was applied using a cyclin D1–specific monoclonal antibody. Cyclin D1 expression was seen both in

the nucleus and in the cytoplasm of untreated CAL27 cells and in cells treated with DMSO for 1 hour (Fig. 4C). However, the level of cytoplasmic expression went down within 30 minutes of curcumin treatment. Nuclear expression was reduced by 1 hour, and the expression was mostly confined to the nuclear membrane after 2 hours of treatment. Thus, the transcription, stability, and/or localization of cyclin D1 were affected with curcumin treatment in CAL27, an aggressive cell line containing high-level expression of cyclin D1.

To show the direct correlation between the expression of NF-κβ and cyclin D1, we analyzed CCL23 cells in the presence of TNF-β, which should increase the nuclear translocation of NF-κβ. The final concentration of TNF-β used was 10 ng/mL. Figure 5 shows increased nuclear expression of NF-κβ in the presence of TNF-β. There was also an increased expression of phospho-IκB and cyclin D1 with TNF-β. Curcumin treatment resulted in reduced expression of phospho-IκB and cyclin D1. This effect was also seen in the presence of TNF-β. In addition, expression of NF-κβ was also reduced following treatment with curcumin and TNF-β. Thus, the combined gel shift and Western blotting studies showed that there was a direct correlation between reduced nuclear expression of NF-κβ and decreased expression of an NF-κβ activated gene, *cyclin D1*, in curcumin-treated cells.

In vivo inhibition of tumor xenografts with curcumin paste. Initially, CCL23 xenografts in nude mice were injected intratumorally with curcumin dissolved in DMSO. There was little to no effect on tumor growth (data not shown), and the

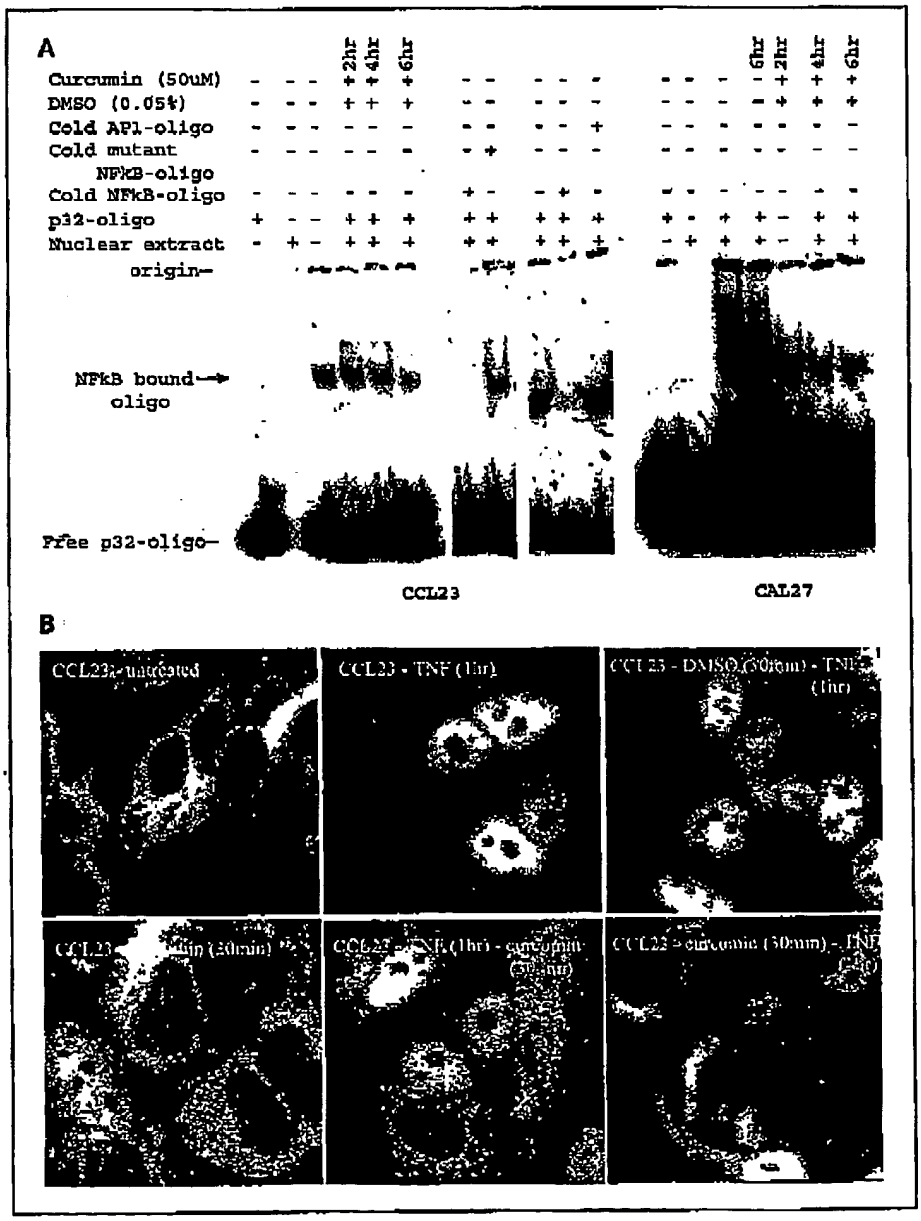

Fig. 3. Reduced NF-κB expression with curcumin treatment. *A*, gel shift assay with the ^{32}P-labeled NF-κB oligonucleotide shows specific binding of NF-κB to the probe. Binding is abolished by the addition of cold NF-κB oligonucleotide and not with mutant NF-κB or activator protein 1 oligonucleotides. There is >50% reduction in the level of NF-κB in both the CCL23 and CAL27 cell lines after a 6-hour treatment with curcumin. *B*, immunofluorescence assay with NF-κB antibody shows the protein to be localized mostly to the cytoplasm (*top left*) and is transported to the nucleus with the addition of TNF-β for 1 hour (*top middle*). Addition of curcumin seems to result in enlargement of nucleus and a modest reduction in cytoplasmic NF-κB (*bottom left*). Effect of curcumin is again modest on nuclear NF-κB after TNF-β treatment (*bottom middle*). Treatment with DMSO before the addition of TNF-β did not have an effect on the level of nuclear NF-κB (*top right*). However, pretreatment with curcumin even for 0.5 hour shows a dramatic decrease in the level of NF-κB (*bottom right*) in 70% to 80% of cells, demonstrating a direct effect on the antiapoptotic transcription factor in HNSCC cell lines.

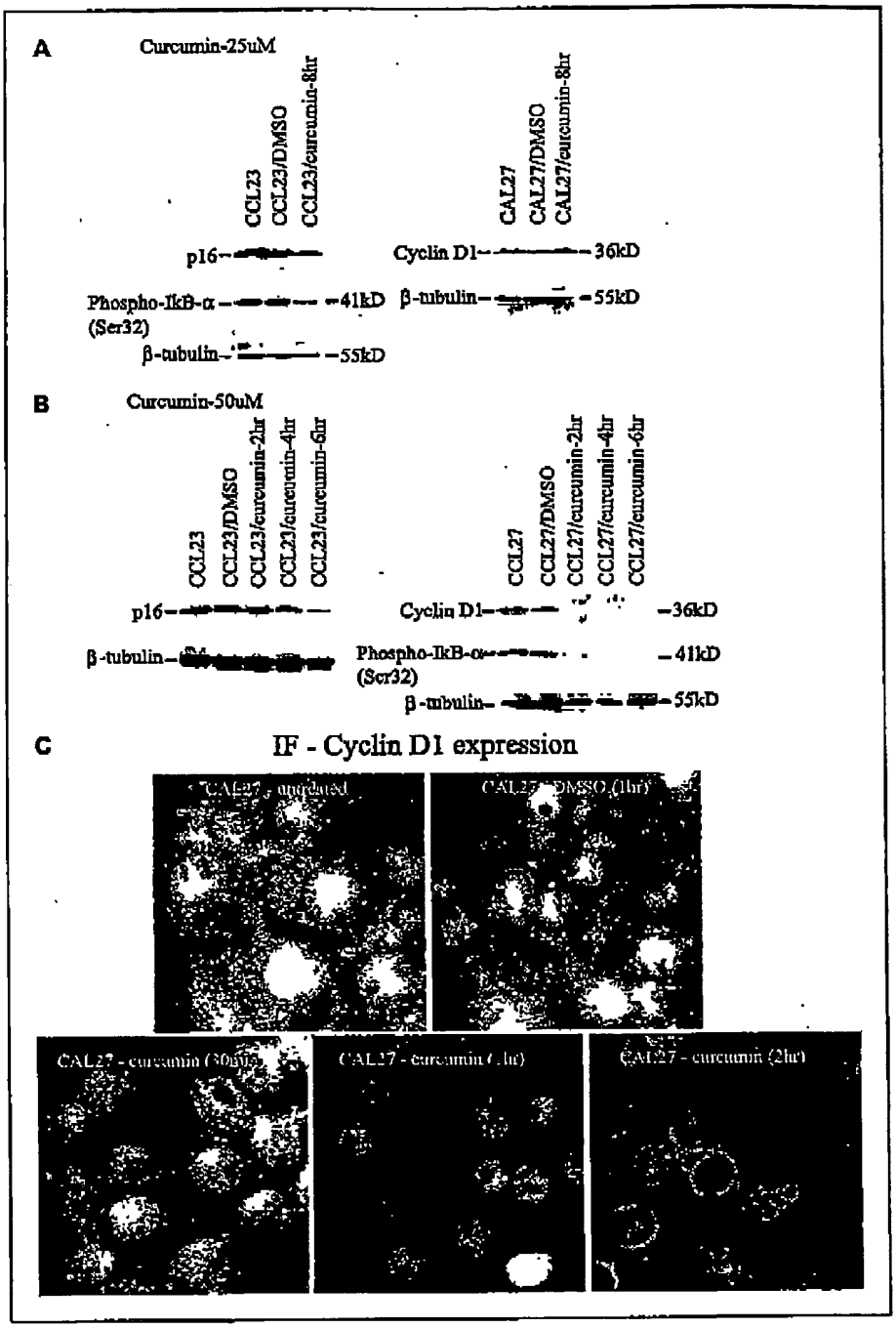

Fig. 4. Decreased expression of cyclin D1 and phospho-IκB-α after curcumin treatment. *A*, the expression of p16 in CCL23 cells and cyclin D1 in CAL27 cells are not affected after 8-hour treatment with 25 μmol/L curcumin. The level of phospho-IF-κB-α is slightly reduced in CCL23 cells. However, as seen in (*B*), expression of cyclin D1 and phospho-IF-κB-α are significantly reduced within 2 hours of curcumin treatment in CAL27 cells. Inhibition of phospho-IκB expression indicates that the cell killing is mediated through the NF-κB pathway. The level of p16 decreases in CCL23 cells at 6 hours posttreatment, possibly reflecting increased cell death by this time period. *C*, immunofluorescence assay shows the level of cyclin D1, both in the cytoplasm and in the nucleus, to be reduced within 1 hour of curcumin treatment compared with untreated cells or cells treated with DMSO. After 2 hours of curcumin treatment, cyclin D1 expression is dramatically reduced and is present mostly in the nuclear membrane. These results confirm the data obtained with the Western blot studies, indicating reduced expression of cyclin D1 possibly due to decreased transcription and increased proteosomal degradation after treatment with curcumin.

delivery of curcumin seemed to be inadequate despite the weekly dose escalation. Both control and curcumin-treated tumors grew at similar rates to a large size by the end of the experimental period. A solution of curcumin/DMSO ranging in concentration from 50 to 250 μmol/L was increased incrementally over 5 weeks. Higher concentrations of curcumin will not differentiate the effect of curcumin from DMSO because the higher concentrations of DMSO required could also result in cell death, as seen in the cells treated with DMSO alone (Fig. 1). An additional problem was that much of the curcumin/DMSO solution leaked out of the injection site and there was significant skin necrosis at that site.

Therefore, to increase the concentration of curcumin applied and to increase absorption through the skin, curcumin was applied as a paste. Curcumin paste is often used as a poultice in India, and there seems to be effective absorption through the skin of hamsters (19). DMSO was used initially, but because of the difficulty in forming a paste with a high concentration of

curcumin, ultimately saline was used for the preparation of curcumin paste. The paste had a thick consistency, and this method allowed topical application of a higher concentration of curcumin onto xenograft tumors. In addition, the paste would remain on the tumor for several hours, allowing enhanced absorption. Xenografts treated with DMSO or saline alone were used as controls. Treatment with the saline/curcumin paste resulted in the inhibition of tumor growth in the xenografts. Growth inhibition was seen in the majority of CAL27 xenograft tumors (Fig. 6). There were 5 mice in the control CAL27 group and 12 mice in the experimental (curcumin-treated) CAL27 group. Tumor growth was observed for 3 weeks. There was a significant decrease in the mean size of the tumors treated with curcumin paste. Modest inhibition of tumor growth was seen in other cell line xenografts (data not shown).

Toxicity studies. There were no significant abnormalities in electrolytes or complete blood counts of curcumin-treated mice, signifying minimal bone marrow or renal toxicity. Sorbitol dehydrogenase, a mouse liver enzyme, was mildly elevated in one animal. There was mild elevation of blood urea nitrogen in some of the mice, indicating possible dehydration; however, creatinine levels were normal. Finally, there were no abnormalities in skeletal muscle, thyroid, salivary gland, pancreas, brain, or gastrointestinal organs. In the livers of a few of the mice, mild subacute bile duct hyperplasia was seen.

Discussion

HNSCC represents 5% of cancers diagnosed annually in the United States. In 2003, 37,200 new cases of head and neck cancers, including skin cancers, were identified in the United States (20). Head and neck cancer refers to a large heterogeneous group of tumors, including the face, nasopharynx, oral cavity, oropharynx, hypopharynx, and/or larynx. The predominant histologic type is squamous cell carcinoma, representing >90% of cases diagnosed each year. Studies have shown predisposing factors to head and neck cancers include chronic tobacco and alcohol use (21). Despite multiple modalities of treatment such as surgery, radiation, and chemotherapy, head and neck cancers continue to have the one of the lowest 5-year survival rates (1). Extensive work has been done to determine if one modality or combination of modalities has any effect on survival. As a consequence of surgery, radiation, and chemotherapy, debilitating outcomes are often observed and experienced by the patients.

Surgery is often the primary treatment in head and neck cancers, followed by postoperative radiation therapy. Patients who are diagnosed with advanced head and neck cancer and undergo surgery are subjected to lengthy, high-risk operations, which often result in functional impairment and disfigurement. Not only are these people physically altered, but their quality of life declines, and survival times may be measured only in months (22–24).

The success of chemotherapy and radiation protocols for organ preservation of laryngeal cancers has led to the increasing use of these protocols for nonlaryngeal HNSCC. Combined chemotherapy and radiation therapy have been implemented for stage III/IV head and neck cancers as an alternative primary treatment modality. Chemotherapy acts as a radiation sensitizer, which improves the tumoricidal activity of radiation. Current standard chemotherapy protocols for HNSCC involve the use of cisplatin and 5-fluorouracil. Radiation is delivered either following chemotherapy or in a concomitant mode. Each of these modalities has multiple associated toxicities, including xerostomia, dysphagia, fever, leukopenia, anorexia, and difficulty in assessing recurrence (25).

The significant morbidity of surgery, radiation, and chemotherapy for HNSCC has led to searches for alternative, less toxic

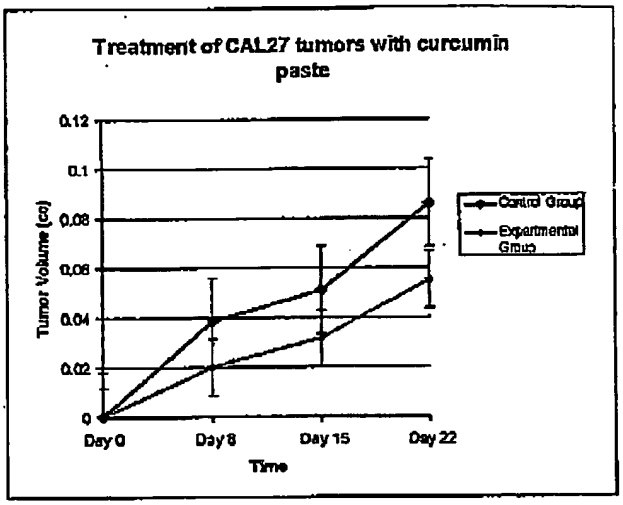

Fig. 6. Inhibition of nude mice xenograft tumors. Application of curcumin as a paste results in a modest growth reduction in CAL27 tumors. There were 12 mice in the experimental group treated with curcumin paste and five mice in the control group treated with saline.

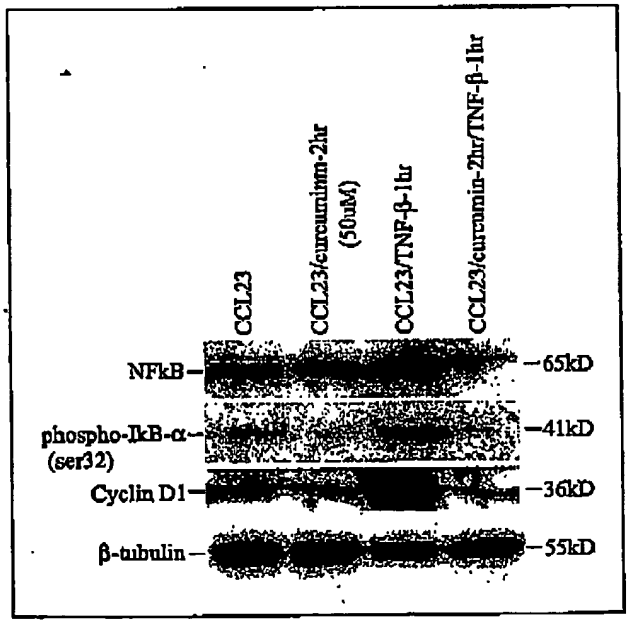

Fig. 5. Western blot demonstrating association between expression of NF-κB and cyclin D1 in CCL23 cells. Treatment with TNF-β results in increased expression of NF-κB, phospho-IκB, and cyclin D1 (*lane 3*) compared with untreated cells (*lane 1*). However, after addition of curcumin, there is reduced expression of phospho-IκB and cyclin D1 (*lane 2*). This effect was also seen even with the addition of TNF-β after curcumin treatment (*lane 4*).

therapies. Tumorigenesis is a result of amplification or overexpression of oncogenes and/or the inactivation of tumor suppressor genes. Thus, novel approaches in treatment of head and neck cancer include the targeting of cell cycle and apoptotic genes (26–28). Alternate therapies are also tried using small molecule inhibitors to target antiapoptotic genes (29, 30). Other therapies, such as the use of green tea, have also been used to inhibit head and neck cancer in animal models. Treatment with green tea, alone or in combination with curcumin, resulted in decreased cell proliferation in head and neck squamous cell lines. When treated with green tea and/or curcumin, cancer cells inhibited the activation of the antiapoptotic transcription factors activator protein 1 and NF-κB (19).

Many studies have shown that curcumin suppresses the proliferation of a variety of tumor cells, including breast, colon, oral, lung, melanoma, myeloma, leukemia, and prostate carcinoma (6–9, 31–39). The mechanism by which curcumin acts is not completely understood. It has many hypothesized actions, including inhibition of proliferation induced by growth factors, suppression of the cell cycle, and induction of apoptosis through mitochondrial-dependent (40, 41) or mitochondrial-independent pathways (6, 42–44). Curcumin also down-regulates the antiapoptotic proteins bcl-2 and bcl-XL, another potential mechanism by which apoptosis is induced (45, 46). We have observed down-regulation of NF-κB and a corresponding decrease in the expression of cyclin D1 with the addition of curcumin. Thus, its growth inhibitory effect is also mediated through its inhibitory activity on the transcription factor, NF-κB.

NF-κB is one of the major activators of transcription. Studies have shown that inhibition of this pathway could result in suppression of tumor growth. One major mechanism of NF-κB activation is through inhibition of IκB phosphorylation. This would result in the retention of NF-κB in the cytoplasm. TNF-β, a major stimulatory factor of NF-kB activation, enhances IκB phosphorylation. However, curcumin could prevent this phophorylation and thus result in reduced NF-κB activation. Our data supports this effect of curcumin in HNSCC through inhibition of the NF-κB pathway. In addition, we also show that inhibition of NF-κB activation is accompanied by reduced cyclin D1 expression. Thus, this supports the effect of curcumin on cell cycle regulation.

The effects of curcumin *in vivo* seem to result from the amount of drug available at the tumor site. Intratumoral injection of curcumin was not effective with the concentrations used in our experiments. Although it is possible to make more concentrated solutions of curcumin and use a smaller volume for injection, higher concentrations of curcumin would also require higher concentrations of DMSO, in which case it may be difficult to differentiate the effect of curcumin from the cytotoxic effects of DMSO. We also observed much leakage of curcumin/DMSO out of the injection site and the development of skin necrosis at the injection site. As a result, the tumor did not retain the volume of curcumin delivered. Therefore, application as a paste seems to be a better alternative. There was observable suppression of *in vivo* tumor growth in the CAL27 xenografts treated with curcumin/saline paste. Data from these experiments suggest that curcumin/saline paste could be effective for HNSCC tumor suppression. The number of animals and tumors was small, however, and further studies are needed to confirm and extend the present study to utilize curcumin as an effective *in vivo* therapy for head and neck cancer.

In conclusion, we have shown that curcumin treatment of head and neck cancer cell lines results in growth inhibition both *in vitro* and *in vivo*. We also show that this growth reduction is mediated through the inhibition of the antiapoptotic transcription factor NF-κB. In addition, we show for the first time that curcumin can be used as a topical paste for growth suppression of HNSCC xenograft tumors. Further studies using a larger set of xenograft tumors and additional cell lines are needed to determine whether curcumin has potential use as a therapeutic or chemopreventive agent for head and neck cancer.

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Liposome-encapsulated curcumin: in vitro and in vivo effects on proliferation, apoptosis, signaling, and angiogenesis.

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BACKGROUND: Because a role for nuclear factor-kappaB (NF-kappaB) has been implicated in the pathogenesis of pancreatic carcinoma, this transcription factor is a potential target for the treatment of this devastating disease. Curcumin (diferuloylmethane) is a phytochemical with potent NF-kappaB-inhibitory activity. It is pharmacologically safe, but its bioavailability is poor after oral administration. METHODS: The authors encapsulated curcumin in a liposomal delivery system that would allow intravenous administration. They studied the in vitro and in vivo effects of this compound on proliferation, apoptosis, signaling, and angiogenesis using human pancreatic carcinoma cells. NF-kappaB was constitutively active in all human pancreatic carcinoma cell lines evaluated and liposomal curcumin consistently suppressed NF-kappaB binding (electrophoretic mobility gel shift assay) and decreased the expression of NF-kappaB-regulated gene products, including cyclooxygenase-2 (immunoblots) and interleukin-8 (enzyme-linked immunoassay), both of which have been implicated in tumor growth/invasiveness. These in vitro changes were associated with concentration and time-dependent antiproliferative activity (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide assay [MTT assay]) and proapoptotic effects (annexin V/propidium iodide staining [fluorescence-activated cell sorting] and polyadenosine-5'-diphosphate-ribose-polymerase cleavage). RESULTS: The activity of liposomal curcumin was equal to or better than that of free curcumin at equimolar concentrations. In vivo, curcumin suppressed pancreatic carcinoma growth in murine xenograft models and inhibited tumor angiogenesis. CONCLUSIONS: Liposomal curcumin down-regulated the NF-kappaB machinery, suppressed growth, and induced apoptosis of human pancreatic

cells in vitro. Antitumor and antiangiogenesis effects were observed in vivo. The experiments in the current study provide a biologic rationale for treatment of patients suffering from pancreatic carcinoma with this nontoxic phytochemical encapsulated in liposomes for systemic delivery. Copyright 2005 American Cancer Society.

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Special Report

Standard classification of rosacea: Report of the National Rosacea Society Expert Committee on the Classification and Staging of Rosacea

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Sections

INTRODUCTION

Rosacea is well recognized as a chronic cutaneous disorder primarily of the convexities of the central face (cheeks, chin, nose, and central forehead), often characterized by remissions and exacerbations. Based on present knowledge, it is considered a syndrome, or typology, encompassing various combinations of such cutaneous signs as flushing, erythema, telangiectasia, edema, papules, pustules, ocular lesions, and rhinophyma.[1] In most cases, some rather than all of these stigmata

appear in any given patient.

Rosacea appears to be quite common, and in an epidemiologic study in Sweden its prevalence was 10%.[2] It has been most frequently observed in patients with fair skin, but has also been diagnosed in Asians and African Americans. Rosacea occurs in both men and women and, although it may occur at any age, the onset typically begins at any time after age 30.[3]

Despite its apparent high incidence, the nosology of rosacea is not well established, and the term "rosacea" has been applied to patients and research subjects with a diverse set of clinical findings that may or may not be an integral part of this disorder. In addition to the diversity of clinical manifestations the etiology and pathogenesis of rosacea are unknown, and there are no histologic or serologic markers.

Therefore, the National Rosacea Society assembled a committee to develop a standard classification system that can serve as a diagnostic instrument to investigate the manifestations and relationships of the several subtypes and potential variants of rosacea. Standard criteria for diagnosis and classification of patients are essential to perform research, analyze results and compare data from different sources, and may further serve as a diagnostic reference in clinical practice. The standard terminology will also facilitate clear communication among a broad range of basic, clinical, and other researchers; practicing dermatologists, primary care physicians, ophthalmologists and other specialists; health and insurance administrators; and patients and the general public.

The committee based the standard classification system on present scientific knowledge and morphologic characteristics. This avoids assumptions on pathogenesis and progression, and provides a framework that can be readily updated and expanded as new discoveries are made. As knowledge increases, it is hoped that the definition of rosacea may ultimately be based on causality, rather than on morphology alone.

The following provisional classification system describes the primary features of rosacea and defines 4 subtypes and 1 variant. Evolution from one subtype to another may or may not occur, and research to investigate this process may provide important insight into the pathogenesis of rosacea. Regardless of subtype, however, each individual characteristic may progress from mild to moderate to severe. Early diagnosis and treatment are therefore recommended.

DIAGNOSTIC CRITERIA

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Primary features

Rosacea typically affects the convexities of the central face. The presence of one or more of the following signs with a central face distribution is indicative of rosacea. These signs are commonly transient, and each may occur independently. Many patients may present with more than one of these diagnostic features.

- Flushing (transient erythema). A history of frequent blushing or flushing is common.
- Nontransient erythema. Persistent redness of the facial skin is the most common sign of rosacea.
- Papules and pustules. Dome-shaped red papules with or without accompanying pustules, often in crops, are typical. Nodules may also occur. Although patients with concomitant acne may exhibit comedones, comedones should be considered part of an acne process unrelated to rosacea.
- Telangiectasia. Telangiectases are common but not necessary for a rosacea diagnosis.

Secondary features

The following signs and symptoms often appear with one or more of the primary features of rosacea, but in some patients can occur independently.

- Burning or stinging. Burning or stinging sensations with or without scaling or dermatitis may occur, especially on malar skin.[4]
- Plaque. Elevated red plaques without epidermal changes in the surrounding skin may occur.
- Dry appearance. Central facial skin may be rough and scaling so as to resemble dry skin and suggest an eczematous dermatitis, and may often include the coexistence of seborrheic dermatitis. This "dryness" may be associated with burning or stinging sensations, and may be caused by irritation rather than the disease process.
- Edema. Edema may accompany or follow prolonged facial erythema or flushing. Sometimes soft edema may last for days or be aggravated by inflammatory changes. Solid facial edema (persisting hard, nonpitting edema) can occur with rosacea, usually as a sequel of the

papulopustular type, and also independently of redness, papules and pustules, or phymatous changes.

- Ocular manifestations. Ocular manifestations are common, and range from symptoms of burning or itching to signs of conjunctival hyperemia and lid inflammation. Styes, chalazia, and corneal damage may occur in many patients with rosacea in addition to cutaneous stigmata. The severity of ocular manifestations may not be proportional to those of the skin.
- Peripheral location. Rosacea has been reported to occur in other locations,[5] but the frequency and occurrence of this are ill-defined. Rosacea in peripheral locations may or may not be accompanied by facial manifestations.
- Phymatous changes. These can include patulous follicles, skin thickening or fibrosis, and a bulbous appearance. Rhinophyma is the most common form, but other phymas may occur (Table I).

Table I. Guidelines for the diagnosis of rosacea

Presence of one or more of the following primary features:
Flushing (transient erythema)
Nontransient erythema
Papules and pustules
Telangiectasia
May include one or more of the following secondary features:
Burning or stinging
Plaque
Dry appearance
Edema
Ocular manifestations
Peripheral location
Phymatous changes

SUBTYPES

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The primary and secondary rosacea features described above often occur together. The most common patterns or groupings of signs are provisionally designated as specific subtypes of rosacea and are described here (Table II). Each subtype includes the fewest signs sufficient to make a diagnosis of the subtype (though not necessarily limited to these), and patients may have characteristics of more than one rosacea subtype at the same time.

Subtype 1: Erythematotelangiectatic rosacea

Erythematotelangiectatic rosacea is mainly characterized by flushing and persistent central facial erythema. The appearance of telangiectases is common but not essential for a diagnosis of this subtype. Central facial edema, stinging and burning sensations, and roughness or scaling may also be reported. A history of flushing alone is common among patients presenting with erythematotelangiectatic rosacea.

Subtype 2: Papulopustular rosacea

Papulopustular rosacea is characterized by persistent central facial erythema with transient papules or pustules or both in a central facial distribution. However, papules and pustules also may occur periorificially (that is, they may occur in the perioral, perinasal, or periocular areas). The papulopustular subtype resembles acne vulgaris, except that comedones are absent. Rosacea and acne may occur concomitantly, and such patients may have comedones as well as the papules and pustules of rosacea. Burning and stinging sensations may be reported by patients with papulopustular rosacea.

This subtype has often been seen after or in combination with subtype 1, including the presence of telangiectases. The telangiectases may be obscured by persistent erythema, papules, or pustules, and tend to become more visible after successful treatment of these masking components.

Subtype 3: Phymatous rosacea

Phymatous rosacea includes thickening skin, irregular surface nodularities, and enlargement. Rhinophyma is the most common presentation, but phymatous rosacea may occur in other locations, including the chin, forehead, cheeks, and ears. Patients with this subtype also may have patulous, expressive follicles in the phymatous area, and telangiectases may be present.

This subtype has frequently been observed after or in combination with subtypes 1 or 2, including persistent erythema, telangiectases, papules, and pustules. In the case of rhinophyma, these additional stigmata may be especially pronounced in the nasal area.

Subtype 4: Ocular rosacea

The diagnosis of ocular rosacea should be considered when a patient's eyes have one or more of the following signs and symptoms: watery or bloodshot appearance (interpalpebral conjunctival hyperemia), foreign body sensation, burning or stinging, dryness, itching, light sensitivity, blurred vision, telangiectases of the conjunctiva and lid margin, or lid and periocular erythema. Blepharitis, conjunctivitis, and irregularity of the eyelid margins also may occur.[6] Meibomian gland dysfunction presenting as chalazion or chronic staphylococcal infection as manifested by hordeolum (stye) are common signs of rosacea-related ocular disease. Some patients may have decreased visual acuity caused by corneal complications (punctate keratitis, corneal infiltrates/ulcers, or marginal keratitis).[7] Treatment of cutaneous rosacea alone may be inadequate in terms of lessening the risk of vision loss resulting from ocular rosacea, and an ophthalmologic approach may be needed.[8]

Ocular rosacea is most frequently diagnosed when cutaneous signs and symptoms of rosacea are also present. However, skin signs and symptoms are not prerequisite to the diagnosis, and limited studies suggest that ocular signs and symptoms may occur before cutaneous manifestations in up to 20% of patients with ocular rosacea. Approximately half of these patients experience skin lesions first, and a minority have both manifestations simultaneously.[9]

Table II. Subtypes and variants of rosacea and their characteristics

	Characteristics
Subtype	
Erythematotelangiectatic	Flushing and persistent central facial erythema with or without telangiectasia.
Papulopustular	Persistent central facial erythema with transient, central facial papules or pustules or both.
Phymatous	Thickening skin, irregular surface nodularities and enlargement May occur on the nose, chin, forehead, cheeks, or ears.
Ocular	Foreign body sensation in the eye, burning or stinging, dryness, itching, ocular photosensitivity, blurred vision, telangiectasia of the sclera or other parts of the eye, or periorbital edema.
Variant	
Granulomatous	Noninflammatory; hard; brown, yellow, or red cutaneous papules; or nodules of uniform size.

VARIANTS

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Variants of rosacea, which do not represent morphologic patterns or combinations as seen in rosacea subtypes, may occur. To date, the committee has recognized one such variant.

Granulomatous rosacea

Granulomatous rosacea is characterized by hard, yellow, brown, or red cutaneous papules or nodules that may be severe and lead to scarring. These lesions tend to be less inflammatory than papules and pustules and sit upon relatively normal-appearing skin. They can vary in size among patients but are monomorphic in each individual patient.

and typically appear on the cheeks and periorificial areas. Granulomatous rosacea may occur in locations other than those in which the phymas are observed. The presence of other rosacea signs is not needed for a diagnosis of the granulomatous rosacea variant.

EXCLUSIONS

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The committee noted that certain disorders may have been prematurely identified as associated with rosacea or as a variant of rosacea, and for clarity should be recognized at this time as separate entities. There is insufficient basis at present to include the following conditions as types of rosacea.

Rosacea fulminans

Popularly known as pyoderma faciale, the grouping of this disorder as a type of rosacea is premature. It is characterized by the sudden appearance of papules, pustules, and nodules, along with fluctuating and draining sinuses that may be interconnecting. The condition appears primarily in women in their 20s, and intense redness and edema also may be prominent.

Steroid-induced acneiform eruption

Steroid-induced acneiform eruption is not a variant of rosacea and can occur as an inflammatory response in any patient during or after chronic corticosteroid use. The same inflammatory response may also, of course, occur in patients with rosacea.

Perioral dermatitis

Although rosacea papules may appear in the perioral area, as noted earlier, perioral dermatitis without rosacea symptoms cannot be classified as a variant of rosacea. Perioral dermatitis is characterized by such stigmata as microvesicles, scaling, and peeling.

FUTURE

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This investigational instrument is intended to set the stage for a better understanding of rosacea and its subtypes among researchers and practitioners by fostering communication and facilitating the development of a research-based classification system. As a provisional standard classification system, it is likely to require modification in the future as the pathogenesis and subtypes of rosacea become clearer, and as its relevance and applicability are tested by investigators and clinicians. The committee welcomes reports on the usefulness and limitations of these criteria.

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The National Rosacea Society is a 501(c)(3) nonprofit organization whose mission is to support rosacea research, including the awarding of research grants, and to provide educational information on rosacea to physicians, patients, and the public. Reports or inquiries should be directed to the National Rosacea Society, 800 S Northwest Hwy, Suite 200, Barrington, IL 60010; telephone 1-888-662-5874; E-mail: **rosaceas@aol.com.**

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Q. Is there any evidence that certain vitamins help control rosacea?

A. Studies have not been conducted to determine whether any vitamin may help control the signs and symptoms of rosacea. However, niacin, one of the B complex vitamins found in certain foods as well as supplements, may act as a vasodilator and consequently induce or intensify flushing. Rosacea sufferers affected by niacin therefore may wish to avoid foods or supplements containing this substance. As a preventive measure, aspirin taken one to two hours before ingesting foods or supplements with niacin may reduce the intensity of the flush.[1]

Q. What is the difference between rosacea and adult acne?

A. Although rosacea has sometimes been referred to as "adult acne," it is a distinctly different disease than acne. The bumps and pimples of adult acne resemble the papules and pustules of subtype 2 rosacea, but there are a number of important differences between the two disorders.

Unlike rosacea, which typically appears in the central facial area, acne often appears on the lateral as well as the central face, especially in older teens. Also, unlike acne, rosacea does not include comedones, commonly known as blackheads. In further contrast to acne, rosacea is usually associated with flushing, and ocular signs and symptoms are frequently present.

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Vascular Growth Factors and Lymphangiogenesis

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Jussila, Lotta, and Kari Alitalo. Vascular Growth Factors and Lymphangiogenesis. *Physiol Rev* 82: 673–700, 2002; 10.1152/physrev.00005.2002.—Blood and lymphatic vessels develop in a parallel, but independent manner, and together form the circulatory system allowing the passage of fluid and delivering molecules within the body. Although the lymphatic vessels were discovered already 300 years ago, at the same time as the blood circulation was described, the lymphatic system has remained relatively neglected until recently. This is in part due to the difficulties in recognizing these vessels in tissues because of a lack of specific markers. Over the past few years, several molecules expressed specifically in the lymphatic endothelial cells have been characterized, and knowledge about the lymphatic system has started to accumulate again. The vascular endothelial growth factor (VEGF) family of growth factors and receptors is involved in the development and growth of the vascular endothelial system. Two of its family members, VEGF-C and VEGF-D, regulate the lymphatic endothelial cells via their receptor VEGFR-3. With the aid of these molecules, lymphatic endothelial cells can be isolated and cultured, allowing detailed studies of the molecular properties of these cells. Also the role of the lymphatic endothelium in immune responses and certain pathological conditions can be studied in more detail, as the blood and lymphatic vessels seem to be involved in many diseases in a coordinated manner. Discoveries made so far will be helpful in the diagnosis of certain vascular tumors, in the design of specific treatments for lymphedema, and in the prevention of metastatic tumor spread via the lymphatic system.

I. INTRODUCTION TO ANGIOGENESIS AND LYMPHANGIOGENESIS

Embryonic vascular development involves a complex series of events during which the endothelial cells differentiate, proliferate, migrate, and undergo maturation into an organized network of vessels (176, 177). The first step in the development of the blood vessels is called vasculogenesis, which is the process where endothelial cells are generated from their mesenchymal precursors and spontaneously assemble into tubules that fuse to form the primary vascular plexus of the embryo. Remodeling and expansion of these primary vessels into arteries, veins, and capillaries of different sizes is called angiogenesis. Although, by definition, vasculogenesis precedes angiogenesis, in practice the two processes continue in parallel during early development. Tissues that are vascularized by vasculogenesis are generally of endodermal origin (lung, pancreas, spleen, heart, and large blood vessels), while tissues of ectodermal and mesodermal derivation (such as the kidney and the brain) are vascularized primarily via angiogenesis.

The oxygen and nutrients supplied by the vascular system are crucial for cell function and survival. In fact, the cardiovascular system is the first organ system to develop in embryos, supplying oxygen and nutrients to the growing tissues. During organogenesis, the proximity of growing cells to the circulation is ensured by the coordinated growth of blood vessels and organ parenchyma. At the same time that the blood vessels form, the precardiac myoblasts develop by differentiating from the mesothelial cells and form the heart (179). On *day 9* of mouse embryonic development (E9), the heart starts to beat and blood starts to circulate through the newly formed network of vessels (147). In the yolk sac blood islands, mesenchymal cells give rise to both endothelial and hematopoietic cells (37). These cells organize into clusters consisting of future endothelial cells in the outer layer surrounding hematopoietic cells. The endothelial cells then coalesce with those of the neighboring blood islands to form a primitive honeycomb-like blood vessel network, and the hematopoietic cells differentiate into erythrocytes. Later, hematopoiesis resumes in the embryo in the para-aortic region (AGM region) and in the fetal liver and finally in the bone marrow.

A complex orchestration of molecular regulators is needed for the blood vessels to grow. The vasculature begins as a plexus of primitive capillary tubes that are subsequently modified to generate the more complex vascular network of adults. Sprouting of new vessels from preexisting ones is the most frequent mechanism of angiogenesis in embryos, and it involves several sequential steps (223). First, the extracellular matrix components surrounding the endothelial cells are degraded locally by proteases produced by the endothelial cells. This allows the chemotactic migration of endothelial cells toward angiogenic stimuli. Subsequently, the endothelial cells in the midsection of the new vessels proliferate and form a lumen, and adjacent sprouts anastomose and form loops, which become perfused with circulating blood. The loops between the vessels can also form by another mechanism called intussusceptive growth, a form of angiogenesis involving the in situ remodeling of the vessels by protruding interstitial tissue columns. In this process, a large sinusoidal capillary can be divided into smaller capillaries, which then grow separately (176).

Although endothelial cells initiate angiogenesis, they cannot complete the process. Newly formed capillary sprouts are fragile and remain susceptible to remodeling as long as they lack appropriate perivascular structures. The maturation of new blood vessels into stable and functional vessels requires the accumulation of a basal lamina and recruitment of pericytes and smooth muscle cells to cover tightly the abluminal side of the vessel (Fig. 1) (93). The smooth muscle cells provide structural support to the larger vessels protecting the vessels against rupture and are important regulators of blood flow and pressure by their contractile abilities (22). Pericytes are absent or only loosely attached to vessels undergoing angiogenesis, suggesting that the mural cells stabilize nascent vessels by inhibiting endothelial cell proliferation and migration and by stimulating extracellular matrix production (19). Under certain conditions the pericytes support endothelial cell survival, for example, in the neonatal retina during hyperoxia-induced regression of the retinal vessels, where associated pericytes spare some of the vascular branches (19).

Smooth muscle cells are separated from the endothelial cells by a basement membrane and thus are not in direct contact with the endothelium. In contrast, the pericytes share their basement membrane with the endothelial cells and make direct contact with them through holes in the basement membrane. In sites where selective filtration is required, like in the kidney glomeruli and lung parenchyma, pericytes are selectively positioned to allow the fluid or gas exchange. Pericytes and smooth muscle cells may be derived from the mesenchyme variously by in situ differentiation, *trans*-differentiation from endothelial cells, from bone-marrow precursors or macrophages, soon after the endothelial cells have formed tubes. Like the endothelial cells, they proliferate and migrate in parallel to the growth of the vascular plexus (19, 37).

The vascular system is a highly heterogeneous and nonuniform organ system. It consists of an arterial and a venous system that differ structurally and functionally. It has been proposed that establishment of the identities of the arterial and venous vasculatures is under the control of related but distinct genetic programs (75, 218, 230).

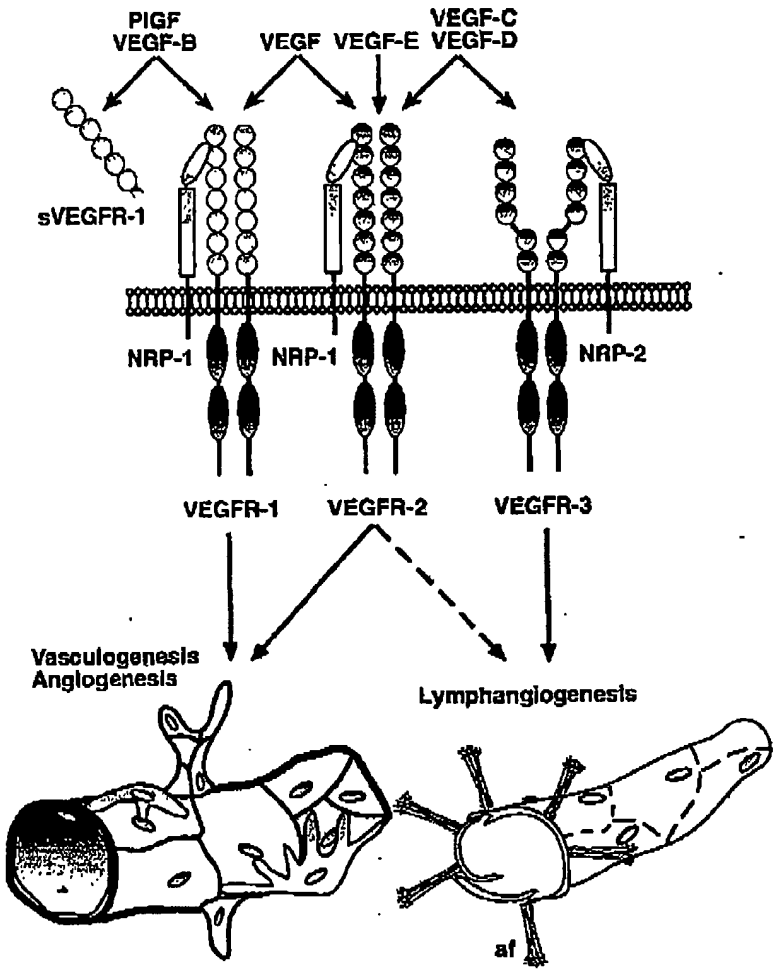

FIG. 1. Receptor binding specificity of vascular endothelial growth factors (VEGFs). VEGFR-1 and VEGFR-2 mediate growth factor signals for blood vascular endothelial cells, whereas VEGFR-3 mainly regulates lymphatic endothelial cells. Neuropilin (NRP)-2 has recently been shown to be coexpressed with VEGFR-3 in the lymphatic endothelium, and VEGFR-2 has occasionally been detected in lymphatic endothelial cells. The permeability of blood vessels is tightly controlled, whereas lymphatic vessels have interendothelial openings that allow relatively free import of fluid and macromolecules. Note that the lymphatic vessels are attached to the interstitial matrix via specific anchoring filaments (af). PIGF, placenta growth factor.

Endothelial cells differ considerably in the arterial, capillary, and venous compartments, and there is further heterogeneity in the different organs (47). Recent molecular probing of the endothelial cell surface by phage display library panning in vivo has revealed striking molecular specificity for the availability of molecular determinants in different vascular endothelia (182). Endothelial cells in different vessels have distinct characteristics, such as fenestrations, cell junctions, enzymes, and carrier systems. For example, fenestrated endothelia are seen in places where extensive molecular exchange occurs across the blood vessel wall, such as in endocrine glands, choroid plexus, and kidney. Differentiation of endothelial cells is dependent on interactions with local parenchymal cells in the target tissues. Although it is not always known which cell type induces the organotypic differentiation of endothelial cells, the existence of such cell-cell interactions seems to be widely accepted.

A. Physiological and Pathological Angiogenesis

Angiogenesis is also required for the maintenance of the functional and structural integrity of tissues during postnatal life. Vasculogenesis is mainly restricted to early development, while new vessels in adults appear to be formed by angiogenesis (37). However, adults are apparently able to mobilize bone marrow-derived endothelial precursor cells for angiogenesis (174). In healthy adults, the endothelial cell turnover is usually very low, and the vascular endothelia are maintained in quiescence by a balance of positive and negative regulators of angiogenesis. Angiogenesis is limited to sites where the metabolic demands of the tissue are such that new blood vessels are needed. In wound healing, fracture repair, inflammation, folliculogenesis, and ovulation during the menstrual cycle, as well as in situations of ischemia, the positive regulators predominate, leading to the activation of an-

giogenic mechanisms (67). Cells suffering from hypoxia start to release angiogenic factors to establish better contact with the circulating blood. Metabolic stimuli, including hypoglycemia and low pH, also participate in the induction of vessel growth, but these mechanisms are less well known. In contrast to developmental angiogenesis, angiogenesis in adults originates mostly in mature blood vessels. In embryos, endothelial cells are loosely connected and actively growing, whereas in adults they are quiescent and encapsulated by a thick mural coat. Therefore, the blood vessels must first become destabilized to allow new growth. A carefully orchestrated activation of several signaling molecules is needed, which may differ in different tissues (37). In contrast to angiogenesis in embryos, there is often inflammation associated with adult angiogenesis, attracting monocytes/macrophages, platelets, mast cells, and other leukocytes.

Angiogenesis results in a higher capillary density, but also the larger vessels are modified by the lack of an adequate oxygen supply. In the case of acute or chronic occlusion of a major artery (coronary, femoral artery), preexisting arteriolar connections can be recruited by arteriogenic mechanisms to bypass the site of occlusion (37). Arteriogenesis produces rapid circumferential growth in the preexisting collateral vessels, which are less perfused with blood under normal flow conditions. These vessels have the ability to dramatically increase their lumen by proliferation of endothelial and smooth muscle cells (33). Recent findings suggest that endothelial precursors in the adult bone marrow also contribute to the expansion of preexisting collaterals (40). As a result of the increased collateral flow, endothelial cells recruit monocytes, which are capable of remodeling the media of the vessel wall. Activated endothelial cells then induce the regrowth of smooth muscle cells in the vessel wall. The smooth muscle cells synthesize a new elastic lamina to the enlarged collaterals. Once the mural cells have been recruited into the vessels, they further muscularize the nascent vasculature by sprouting or by migrating longitudinally along preexisting vessels. After a couple of months the new collateral artery is almost indistinguishable from a normal artery (37). Arteriogenesis differs from angiogenesis in several aspects, but the two processes also share certain mechanisms. For example, angiogenic factors, like fibroblast growth factor (FGF), can activate both mechanisms, but angiogenesis is induced by hypoxia whereas main driving force in arteriogenesis is inflammation (33). Arteriogenesis can counteract the damage in ischemic tissues, such as heart, brain, or limbs, and growth factors that are involved in the arteriogenesis hold great promise for treatment of patients with ischemic diseases, especially those that are poor candidates for mechanical revascularization or bypass surgery.

One of the most extensively studied forms of pathological angiogenesis is tumor angiogenesis (68). Like normal cells, tumor cells need to be located at a close distance from the blood vessels serving the metabolic demands of the growing tumor. The stage in tumor development when a solid tumor grows beyond a few millimeters in diameter and starts to generate its own microcirculation is called the angiogenic switch (70). It means the transition of an avascular tumor to a tumor with its own blood supply. At this stage the endothelial cells transit from a quiescent into an angiogenic state; the positive regulators are induced. Negative regulators can also decrease; for example, trombospondin levels decrease in tumors upon loss of the p53 tumor suppressor gene (28, 91).

Tumor blood vessels are leaky and immature, at least partly because the pericytes and smooth muscle cells are usually poorly recruited to the tumors. The vessels are multilayered, protrude extensions bridging and splitting vessels, contain intercellular and transcellular holes, show relatively uncontrolled permeability, and undergo constant remodeling (68). These vessels resemble angiogenic vessels in other settings, such as in wound healing, with the exception that tumor vessels do not mature properly. Tumor angiogenesis is said to resemble a physiological response that is initiated but not terminated and is considerably more chaotic (53). Occasionally some of the endothelial cells in the tumor vessels are replaced by tumor cells, forming so-called mosaic blood vessels (44). Angiogenesis also takes place in other pathological conditions such as proliferative retinopathy, rheumatoid arthritis, psoriasis, and juvenile hemangioma (67).

B. Lymphangiogenesis

Lymphatic vessels are also part of the vascular circulatory system. The lymphatic system is made up of an extensive network of capillaries, collecting vessels, and ducts that permeate most of the organs (183). Unlike the blood vasculature which forms a continuous loop, the lymphatic system is an open ended, one-way transit system. These vessels collect the extravasated protein-rich fluid and lymphocytes from the tissues and transport them back into the circulation. From the lymphatic capillaries, the fluid is transferred to the collecting lymphatic vessels and ultimately into the venous circulation via the thoracic duct. Larger lymphatic vessels are surrounded by a muscular layer that contracts automatically when the vessel becomes stretched with fluid. In addition, external factors such as skeletal muscle contractions or arterial pulsation compresses the vessels and increases the efficiency of fluid transport.

In tissue sections, lymphatics resemble blood vessels but are generally thinner walled and more irregular. Lymphatics have a low intraluminal pressure and contain a bloodless fluid called lymph, which consists of interstitial

tissue fluid, macromolecules, and cells (227). The lymphatic vessels were discovered in 1627 by Gasparo Asellius (11), at about the same time that the blood circulation was described by William Harvey (89). However, compared with blood vascular studies, the lymphatic system has remained relatively neglected until the present day. This has been partly due to the difficulties in recognizing these vessels in tissues, due to a lack of specific markers. The only way of differentiating the lymphatic vessels has been based on morphology in histological samples and infusions of colored dyes, contrast agents, or macromolecules into the tissues. However, within the past few years molecules expressed specifically in the lymphatic endothelial cells have been found.

The lymphatic vessels also form part of the immune system by continuously transporting the white blood cells within the lymphoid organs (spleen, tonsils, thymus, Peyer patches, and lymph nodes) and bone marrow and transporting antigen-presenting cells. Mononuclear phagocytes and also lymphocytes patroling the tissues enter the afferent lymph vessels and the lymph nodes to elicit primary immune responses before reentering the vasculature. Endothelial receptors and binding proteins are involved in this trafficking of specific lymphatic cell populations.

Lymphatic vessels start to develop in embryos around midgestation, in parallel with the development of blood vessels and most of the organs. When the embryo grows, these vessels are needed for the regulation of the interstitial tissue pressure. The origin of the lymphatic vessels has long been controversial. Historically, the best accepted view of lymphatic development is the one proposed by Sabin (184, 185). On the basis of the findings from her injection experiments, Sabin proposed that early in fetal development, isolated primitive lymph sacs originate by endothelial cell budding from embryonic veins. The two jugular lymph sacs develop from the junction of the subclavian and anterior cardinal veins. Later in the development, the rest of the lymph sacs originate from the mesonephric veins, the veins of the Wolffian bodies, the primitive inferior vena cava and the junctions of the primitive iliac veins and the posterior cardinal veins. Sabin's model proposes that the peripherial lymphatic system then spreads from these primary lymph sacs by endothelial sprouting into the surrounding tissues and organs where local lymphatic capillaries form.

An alternative model has suggested that the initial lymph sacs arise in the mesenchyme from precursor cells ("lymphangioblasts"), independent of the veins and secondarily establish venous connections (97). Although recent reports about the development of the lymphatic vessels support Sabin's theory (52, 222), the existence of primitive lymphangioblasts, which can be recruited by the developing lymphatic vessels, has been shown at least in avian species (189). One should thus note that a combination of the two mechanisms is possible, whereby centrifugally sprouting lymphatic vessels anastomose with lymphatics developing from lymphangioblasts in tissues.

The lymphatic vessels differ in many ways from the blood vessels, but they also share many properties. Both vascular systems are lined by the endothelium, and the larger vessels are supported by a smooth muscle framework, particularly around luminal valves, which are present in the veins and in the large lymphatics (227). Both have vasa vasorum, the blood vessel network providing nutrition for the vessel wall. The smooth muscle layer in blood vessels controls the contractile tone of the vessels in response to vasoactive substances. Blood vessels have a continuous or fenestrated basement membrane and tight interendothelial junctions, which make the vessel wall selectively permeable to cells, fluids, and molecules, whereas lymphatic vessels have a relatively free import for interstitial fluid. Lymphatic endothelial cells have complex overlapping intercellular junctions and specialized anchoring filaments, which hold the vessel open as tissue pressure rises (227). It has been suggested that these properties provide the lymphatics a second valvular function, which permits fluid to enter from the interstitium into the initial lymph channels but prevents escape back out into the tissue (210). Liquid, macromolecules, and migrating cells pass through the blood capillary endothelia, enter the tissues, and are gradually absorbed into the lymphatic system. The fluid is transported via the lymphatic capillaries into the collecting vessels and through the lymph nodes, returning eventually to the circulation.

The blood vascular endothelium is a relatively leakproof, nonthrombogenic surface, with tightly regulated flow and intraluminal pressure, whereas the lymphatics in contrast are a low flow, a low-pressure system in close contact with the extracellular matrix. Lymph fluid does not contain red blood cells or platelets and is therefore much less coagulable than blood. The large lymphatic vessels with their smooth muscle have intrinsic contractility, which serves as a critical pumping force transporting lymph centrally toward the great veins. Compared with blood capillaries, the lymphatic capillaries send out fewer sprouts, anastomose less frequently, and show much less tendency to retract than undergo changes in size or form (227).

After the discovery of specific molecules regulating the lymphatic vessels, their role in certain pathological conditions has been extensively studied. Abnormal function of the lymphatic vessels is implicated in diseases such as lymphedema, inflammation, infectious and immune diseases, fibrosis, ascites, and tumors such as Kaposi's sarcoma and lymphangioma/lymphangiomatosis. Perhaps most importantly, the lymphatic vessels are involved in tumor metastasis (reviewed in Refs. 110, 167). The identification of factors that promote tumor lym-

phangiogenesis and may promote the growth and spread of tumors via the lymphatic system is important for the development of new, effective treatments for cancer (108, 110, 145, 197, 201).

II. MOLECULAR REGULATION OF BLOOD AND LYMPHATIC VESSELS

Intercellular signaling mechanisms that govern the formation of blood and lymphatic vessels have emerged relatively recently. The complexity of endothelial cell development indicates that its regulation must involve many developmental and tissue-specific differentiation factors. Angiogenic signals are mediated by a number of growth factors and cytokines, and the balance between the positive and negative regulators maintains the adult vessels in a quiescent state (reviewed in Ref. 87). Whenever the balance is disturbed, the vessels react either by activating the angiogenic responses or regress by apoptosis when sufficient growth signals are not present. Interaction of angiogenic growth factors with their target cells triggers a cascade of steps, leading to the formation of blood vessels. Less is known about the regulation of the lymphatic vessels, although similar mechanisms seem to be involved.

Blood vessel development depends on members of the vascular endothelial growth factor (VEGF) family of proteins (Fig. 1). This family consists of VEGF, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PlGF), which bind and activate cell surface receptor tyrosine kinases. Key signals regulating embryonic cell growth and differentiation, as well as remodeling and regulation of adult tissues, are mediated by the tyrosine kinase receptors (75, 109). The VEGF receptor (VEGFR) family includes VEGFR-1 (also known as Flt-1), VEGFR-2 (Flk-1), and VEGFR-3 (Flt4). Neuropilins 1 and 2 (NRP-1/-2) are another class of high-affinity nontyrosine kinase receptors for VEGFs on endothelial and neuronal cell surfaces (157, 169). Recently, additional molecules similar to VEGF and capable of increasing capillary permeability were found in snake venom, suggesting that the family may be even larger (79, 120). The receptors have partly overlapping but independent roles in the vascular development and maintenance, and the expression level of these genes modulates the abundance of different types of vessels in tissues. Other factors that are involved in the regulation of blood and lymphatic vessels are the angiopoietins and Tie-receptors, ephrins, and platelet-derived growth factors (PDGFs), which all act together in a coordinated manner during vessel formation (23, 37, 75). Interestingly, certain highly differentiated endothelia may have additional structurally unrelated regulators, such as EG-VEGF (127).

The regulation of blood vessels has been studied extensively over the past 10 years, but the molecular mechanisms behind lymphatic vessel growth have only been studied since 1995, when VEGFR-3, the first specific growth factor receptor of the lymphatic vessels, was found (105). Thereafter, a wealth of new information about the regulation of the growth of the lymphatics has been gained, and the factors known to regulate blood vessels have also been shown to be involved in the biology of the lymphatic vessels. Angiogenesis and lymphangiogenesis are thought to occur in parallel during embryonic development, and both may be important in adult physiological and pathological conditions. It is thus essential to understand the development and regulation of blood vessels to understand the biology of the lymphatic vessels. One must also consider blood and lymphatic vessels as collaborating parts of the circulatory system.

A. VEGF in Vasculogenesis and Angiogenesis

VEGF, discovered in 1989, is a major mediator of both vasculogenesis and angiogenesis (reviewed in Ref. 61). In endothelial cells, VEGF mediates mitogenic signals by activating VEGFR-1 and VEGFR-2 (62). VEGF is expressed as several isoforms consisting of polypeptides of different sizes (121, 145, 165, 183, 189 and 206 amino acid residues), which are all formed from the same gene by alternative splicing and differ in their ability to interact with extracellular matrix components and with NRP-1 (114, 130, 199, 209). These isoforms are thought to have distinct but overlapping functions in angiogenesis. VEGF is also known as vascular permeability factor, as it promotes the extravasation of fluid and plasma proteins, including fibrin, from the blood vessels (54, 190). The increase in microvascular permeability and tissue deposition of fibrin is considered to enhance the migration of endothelial cells in the extracellular matrix (55).

Two independent studies have shown that VEGF is essential for embryonic vasculogenesis and angiogenesis. Inactivation of only a single VEGF allele in mice resulted in embryonic lethality due to defective angiogenesis (38, 64). Also a reduced number of hematopoietic cells was observed. In mutant mice lacking the 164 and 188 amino acid isoforms of VEGF, one-half of the mice did not survive due to defects in, for example, postnatal angiogenesis in the myocardium, suggesting that the other forms of VEGF cannot completely replace the action of the others (42). Further studies suggest that in particular the heparin binding VEGF isoforms are required for efficient angiogenic sprouting and vascular morphogenesis (D. Shima, personal communication). Partial inhibition of VEGF by a soluble extracellular form of VEGFR-1 resulted in increased mortality and impaired organ development in the early postnatal period (81). It was shown

that in addition to proliferation VEGF is also required for the survival of endothelial cells. Consistent with this, other studies have also shown that VEGF supports the survival in endothelial cells and induces the expression of antiapoptotic proteins in endothelial cells (7, 18, 82).

VEGF is a major regulator of abnormal angiogenesis (reviewed in Ref. 61). Consistent with this, the expression of VEGF is potentiated in response to hypoxia and by activated oncogenes as well as by a variety of cytokines (85, 175, 193). VEGF is important in the etiology of several diseases characterized by pathological angiogenesis such as psoriasis, rheumatoid arthritis, and proliferative retinopathy. Deregulated VEGF expression contributes to the development of solid tumors by promoting tumor angiogenesis (67). Tumor inhibition studies with neutralizing anti-VEGF antibodies suggest that other angiogenic factors may also be involved (117). However, the VEGF signaling pathway is currently considered to be one of the most promising targets for the inhibition of tumor angiogenesis.

B. Role of VEGF-B in the Myocardium

VEGF-B is structurally closely related to VEGF and binds one of its receptors, VEGFR-1 (155). It has two splice variants of which the 167 amino acid form binds to heparan sulfates and NRP-1, while the other, of 186 amino acid residues, is a freely secreted, soluble, and *O*-glycosylated product (143). If the 186 amino acid form is proteolytically cleaved, NRP-1 binding epitopes are exposed in this isoform as well. Both VEGF-B isoforms are able to form heterodimers with VEGF, and perhaps with other growth factors. This adds diversity to their biological roles by allowing a variety of combinations for cellular signal transduction. During development, VEGF-B may modulate the biological activities of VEGF, either by forming heterodimers or by controlling the bioavailability of VEGF (156).

VEGF-B is produced in large quantities by the developing myocardium and by muscle, bone, pancreas, adrenal gland, and the smooth muscle cell layer of several large vessels, but not by endothelial cells (1). VEGF-B is likely to act in a paracrine fashion as its receptor is almost exclusively located on endothelial cells. VEGF-B is a very weak endothelial cell mitogen when produced in mammalian cells (155), but otherwise its biological role is still unclear. It is possible that some of the biological activity of VEGF-B produced in mammalian cells can be attributed to VEGF/VEGF-B heterodimers.

Mice lacking a functional VEGF-B gene are healthy and fertile, but depending on the genetic background may have a first degree heart block (conduction defect) or reduced heart size (2, 17). The knockout mice display a striking vascular dysfunction after coronary occlusion, and they show impaired recovery from experimentally induced myocardial ischemia (17). Considering such results, it is interesting to note that while VEGFR-1 and VEGFR-2 were expressed rather uniformly in the developing vasculature, only VEGFR-1 was prominently expressed in the human fetal coronary endothelium (166). These results suggest a role for VEGF-B in the coronary vasculature and potential clinical use in therapeutic angiogenesis.

C. PlGF Is Essential in Pathological Angiogenesis

PlGF was discovered in the human placenta, and it is ~50% homologous to VEGF (139). Three splice isoforms of PlGF have been published, and PlGF-2 at least competes with $VEGF_{165}$ for binding to VEGFR-1 (35, 140, 163). This is considered to increase the proportion of VEGF available to activate VEGFR-2, thereby potentiating the angiogenic properties of VEGF (163). A lack of PlGF has no effect on embryonic development, even in combination with a loss of VEGF-B (41). However, loss of PlGF impairs angiogenesis associated with tumors, ischemia, myocardial infarcts, and experimental retinopathy and leads to prolonged healing of incisional skin wounds (41). During collateral growth after ligation of the femoral artery, PlGF was found to be essential for plasma extravasation, monocyte recruitment, and the growth of endothelial and smooth muscle cells. These results indicate that PlGF activates membrane-bound VEGFR-1 and specifically potentiates the angiogenic response to VEGF. In contrast to the essential role of VEGF in physiological and pathological angiogenesis, the role of PlGF is restricted to pathological vessel formation and is therefore a possible target for therapy.

PlGF is not needed to enhance VEGF signaling in embryonic vascular development. This is probably because VEGF is upregulated in response to a lack of PlGF. This suggests that PlGF serves as an inert regulator of the VEGF activity during development. The need for amplification of VEGF responses in adult pathological angiogenesis might be explained by the requirement of stronger responses to VEGF than in embryonic angiogenesis (41). When new blood vessels form in adults, endothelial cells may become more responsive to VEGF by upregulating PlGF and VEGFR-1. PlGF and VEGFR-1 are minimally expressed in normal quiescent adult vasculature, but both are markedly upregulated in pathological conditions. Also, the membrane localization of VEGFR-1 is increased in pathological angiogenesis compared with embryonic angiogenesis. PlGF is also a chemoattractant for inflammatory cells, which are hallmarks of pathological angiogenesis and collateral growth. PlGF may contribute to vessel growth in adults by mobilizing bone marrow-derived mononuclear cells. There may also be a synergism between PlGF and VEGF-B in pathological angiogenesis.

D. VEGF-C and VEGF-D Coordinate the Development of Blood Vascular and Lymphatic Endothelia

VEGF-C was cloned from human prostate carcinoma cells, and its mature form consisting of the VEGF homology domain is 30% identical to $VEGF_{165}$ (101). VEGF-C is synthesized as a preproprotein and from which a stepwise proteolytic processing generates several forms, with sequentially increasing binding and activity for its receptors, VEGFR-2 and VEGFR-3 (102). Like VEGF, VEGF-C stimulates the migration of endothelial cells and increases vascular permeability and endothelial cell proliferation but at higher concentrations than VEGF. These signals for endothelial cells are probably mediated through VEGFR-2 in blood vascular endothelial cells, and generally via VEGFR-3 in the lymphatic endothelial cells (102, 105). Unlike VEGF, the expression of VEGF-C does not appear to be regulated by hypoxia (58) but is increased in response to proinflammatory cytokines, suggesting a role in inflammatory responses (178). VEGF-C, along with VEGFR-3, is also prominently expressed by activated macrophages (196; S. Mustjoki, personal communication). The pattern of VEGF-C expression in embryos suggests that it plays a role in the development of the lymphatic vessels, since a paracrine expression pattern is seen between VEGF-C and VEGFR-3 at sites where the first lymphatic sprouts occur (123). Conversely, VEGF-C is already expressed before the emergence of the lymphatics, which also suggests its involvement in vasculogenesis/angiogenesis during early development.

VEGF-C can regulate physiological and pathological blood vessel growth in vivo. It is able to stimulate angiogenesis in the mouse cornea and in the hindlimb ischemia model (36, 228). On the other hand, VEGF-C has been shown to regulate the growth of lymphatic vessels in various experimental models. Overexpression of VEGF-C in skin keratinocytes leads to dermal lymphatic vessel hyperplasia (Fig. 2, *A* and *B*) (100). Signaling via VEGFR-3 alone was shown to be sufficient for the hyperplasia, since transgenic mice overexpressing a mutant form of VEGF-C, which has lost its capacity to bind VEGFR-2 and only binds and activates VEGFR-3 (VEGF-C156S), was able to induce a similar phenotype (216). VEGF-C was also studied in the mature, differentiated chorioallantoic membrane (CAM), which contains lymphatic vessels mainly around arterioles and veins (154). In this assay, VEGF-C acts as a highly specific lymphangiogenic factor. However, when VEGF-C was applied to the early CAM, where the lymphatics have not yet developed, it promoted angiogenesis. The angiogenic versus lymphangiogenic responses to VEGF-C may depend on the degree of proteolytic processing of its precursor and on the expression of it receptors in the blood versus lymphatic endothelial cells of the target tissue. VEGF-C also has synergistic

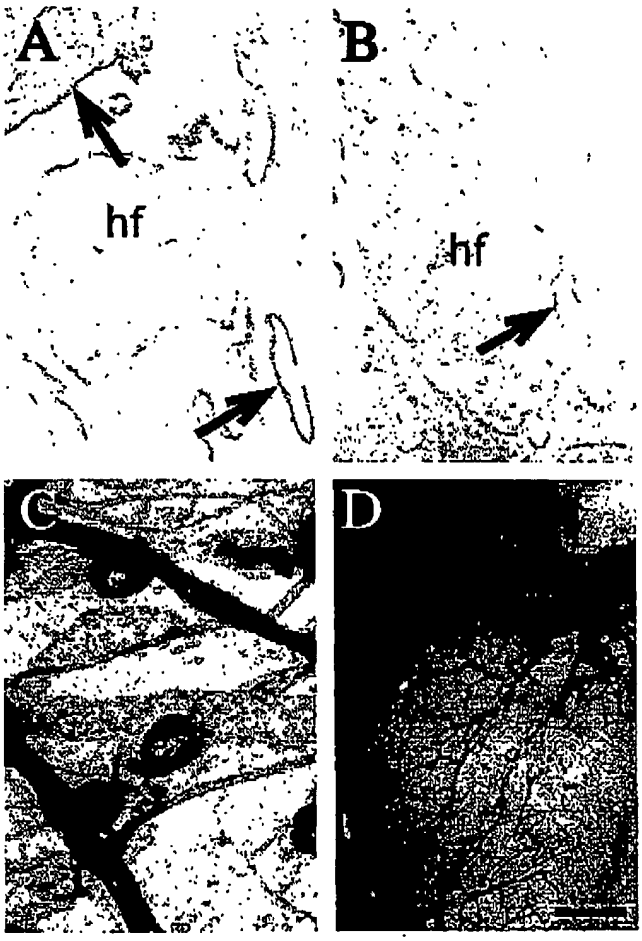

FIG. 2. Lymphatic vessels in adult tissues. Overexpression of VEGF-C in the skin keratinocytes in K14-VEGF-C transgenic mice leads to hyperplasia of lymphatic vessels around the hair follicles (hf) (*A*), compared with the skin of wild-type mice (*B*). The lymphatic endothelium is visualized by immunohistochemical staining for VEGFR-3 (arrows). Lymphatic vessels in the skin (*C*) and pericardium (*D*) of the VEGFR-3-LacZ mice are shown. Lectin perfusion-stained blood vessels appear brown in *C*. Scale bar in *D* is for *A–D*; *A* and *B*, 50 μm; *C*, 30 μm; *D*, 600 μm.

effects with VEGF, during the induction of angiogenesis, and this effect is more prominent in cells expressing both of its receptors (168). In addition, VEGF-C can compete with VEGF in binding to VEGFR-2.

VEGF-D (also known as c-*fos*-induced growth factor or FIGF) is the most recently discovered member of the mammalian VEGF family (3). It shares 61% sequence identity with VEGF-C, and these two growth factors bind to the same receptors on human endothelial cells. VEGF-D is proteolytically processed similarly to VEGF-C, and the proteolytic processing also appears to regulate VEGF-D biological activity and receptor specificity (202). Interestingly, in mice, VEGF-D binds only to VEGFR-3, suggesting that VEGF-D may have a somewhat different function in

mouse and human (12). This is uncommon within the VEGF family as these homologous and evolutionary conserved growth factors are assumed to exhibit similar receptor binding characteristics in different species.

VEGF-D has been shown to be able to stimulate the proliferation of endothelial cells, and it shows angiogenic properties in vitro and in vivo (146). Like VEGF-C, it was also shown to be lymphangiogenic when overexpressed in skin keratinocytes (216). Little is known about the expression of VEGF-D in physiological conditions, but its mRNA has been observed in the developing melanocytes and fibroblasts, lung mesenchyme, and the adult vascular wall (4).

The exact roles of VEGF-C and VEGF-D during embryonic vascular development are still unknown due to the lack of gene deletion studies. In adults, VEGF-C and VEGF-D may regulate the responses of the lymphatic vessels in inflammatory processes and in the regeneration of tissues after trauma, but they may also have important roles in physiological and pathological angiogenesis in various conditions. VEGF-C and VEGF-D may also affect the fluid dynamics in lymphatic vessels and be involved in the formation of valves and recruitment of smooth muscle cells to the developing lymphatic collecting vessels. Unpublished data indicate that VEGF-C and VEGF-D can heterodimerize (M. Jeltsch, personal communication), as has been reported for PlGF and VEGF as well as VEGF-B and VEGF, making their biological properties even more diverse (34, 156).

E. Orf Virus VEGF-E Promotes Angiogenesis

A VEGF homolog, VEGF-E, was recently discovered in the genome of the parapoxvirus, Orf virus, that infects sheep, goats, and occasionally humans (138). Infection by this virus causes proliferative skin lesions in which extensive capillary proliferation and dilation are prominent histological features. Several strains of the virus encode different VEGF-E variants, which bind specifically to VEGFR-2 and NRP-1 and are able to stimulate endothelial cell mitogenesis and vascular permeability (153, 225). VEGF-E is not essential for viral replication but rather plays an important role in modulating the host environment during infection.

F. VEGF Receptors in Endothelial Cell Proliferation, Migration, and Survival

All VEGFRs are characterized by seven extracellular immunoglobulin homology domains (Ig) (Fig. 1), of which the second and third are critical for ligand binding and the first three domains are necessary for establishment of full binding affinity (15, 49, 73, 142). In VEGFR-3, the fifth Ig homology domain is proteolytically cleaved, but the fragments remain together via disulfide bonds. These receptors control many aspects of vascular growth and have partially overlapping expression patterns in the developing vasculature in embryos but show a more restricted expression in adults.

VEGFR-1 and VEGFR-2 are important in blood vascular endothelial cell proliferation, migration, and survival. Mice carrying a homozygous disruption in either of the two VEGF receptors die during early development due to defects in both vasculogenesis and angiogenesis. Embryos lacking functional VEGFR-2 die without mature endothelial or hematopoietic cells (191). Primitive hematopoietic and endothelial progenitors arise normally within the yolk sac blood islands from precursors cells that express VEGFR-2. In contrast, VEGFR-1-deficient mice have normal hematopoietic progenitor cells and endothelial cells that migrate and proliferate but do not assemble into tubes and functional vessels (71). More recent studies have shown that an excessive proliferation of endothelial progenitors is the main factor leading to this disorganization (72). This supports the view that VEGFR-1 is a negative regulator of VEGF-induced vasculogenesis in embryos.

Although VEGFR-1 alone has been shown to induce weak mitogenic signals in vitro (125), it is thought that VEGFR-2 is the major receptor transducing the effects of VEGF in endothelial cells. For example, VEGF-E and site-directed mutants of VEGF, which bind only to VEGFR-2, stimulate endothelial cells similarly to VEGF (82, 116, 148, 225). VEGF also provides survival signals for endothelial cells via VEGFR-2 (82). Outside of the vascular system, VEGFR-1 is expressed in monocytes and macrophages, placental throphoblasts and renal mesangial cells, and VEGFR-2 in hematopoietic stem cells, megakaryocytes, and platelets and retinal progenitor cells (14, 45, 46, 112, 232). Despite the importance of these receptors during embryonic blood vessel development, VEGFR-1 and VEGFR-2 appear to be downregulated in the quiescent adult endothelium.

VEGFR-3 was cloned from a human leukemia cell line and human placenta (76, 161). Two isoforms of VEGFR-3 have been described, designated VEGFR-3s (short) and VEGFR-3l (long), which differ as a result of alternative splicing. The long form is the predominant form in most tissues. An endogenous retroviral genome appears responsible for the short isoform in humans, but this form is missing from mice (96). In adults, the expression of VEGFR-3 is mainly restricted to lymphatic endothelial cells and in hematopoietic cells of monocytic lineage, where it serves as a molecular marker for these vessels (Fig. 2, *C* and *D*) (103, 105). In embryos VEGFR-3 is initially expressed in all vasculature, but during development its expression in blood vessels decreases and becomes restricted to the developing lymphatic vessels (Fig. 3) (105). VEGFR-3-deficient embryos die as a result

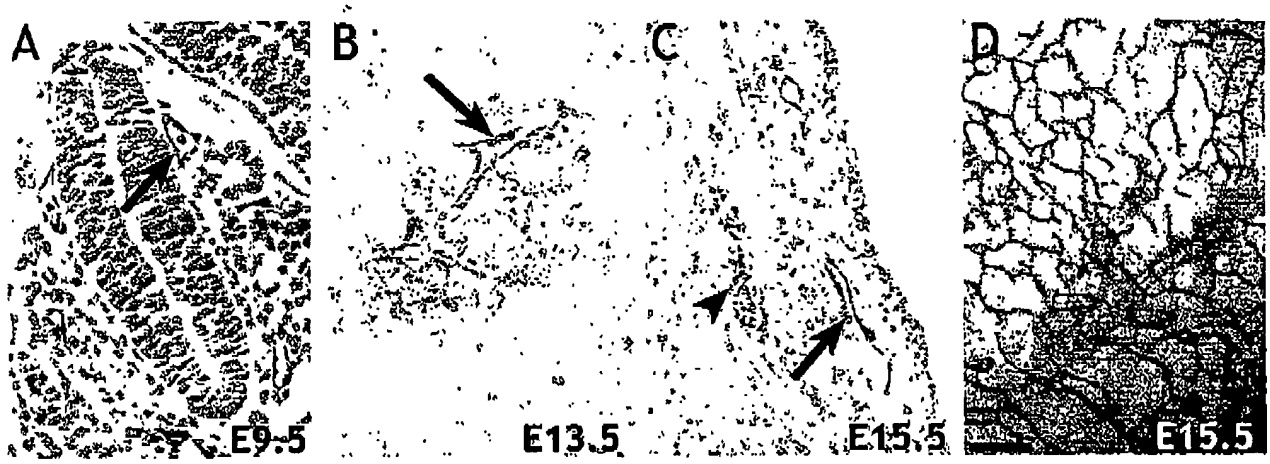

FIG. 3. VEGFR-3 expression during the development of the lymphatic vessels. Blood vascular and lymphatic vessels can be visualized in stained sections from VEGFR-3 knock-out heterozygous embryos (VEGFR-3-LacZ), in which the marker gene reveals sites of VEGFR-3 expression (blue). Early in mouse development (embryonic day 9.5, E9.5) VEGFR-3 is expressed uniformly in the whole developing vasculature (*A*). After the blood vessels have been established, the VEGFR-3-positive lymphatic vessels (arrow) start to sprout from preexisting veins (*B*). Later, VEGFR-3 expression is further downregulated in the blood vessels (arrowhead in *C*) and becomes restricted to the lymphatic vessels (arrow in *C*). The lymphatic vessel network in the skin of E15.5 mouse embryo is shown in whole mount staining (*D*). Scale bar in *D* is for *A–D*; *A*, 30 μm; *B*, 100 μm; *C*, 125 μm; *D*, 80 μm.

of a defect in the remodeling of the primary vascular network and cardiovascular failure at midgestation, before the lymphatic vessels start to develop (52). Interestingly, the differentiation of endothelial cells, the formation of the primary vascular plexus, and vascular sprouting were not disturbed by the absence of VEGFR-3 signaling, but the embryos had severe anemia due to impaired yolk sac hematopoiesis (86). These results suggest that VEGFR-3 plays a dual role, in embryos in cardiovascular development before the emergence of the lymphatic vessels and in adults in the regulation of the lymphatic vessels.

The majority of blood vessel endothelial cell populations around midgestation are positive for both VEGFR-2 and VEGFR-3, suggesting that these receptors have essential roles in angiogenesis (86). Also, VEGFR-1 is seen in the developing vasculature at this stage (71). Endothelial cells are apparently activated by both VEGF and VEGF-C at this stage, but it is not known whether these receptors transduce similar signals. The VEGF/VEGFR-2 system appears to be responsible for most of the growth signals for vascular endothelial cells, but it has also been proposed that VEGF-C induces proliferation of embryonic vascular endothelial cells through VEGFR-2. VEGF-C signaling through VEGFR-2 and VEGFR-3 may thus have distinct roles in embryogenic vasculogenesis (86). In addition, in VEGF-deficient mice, some endothelial cells survive, and this may be promoted by VEGF-C.

In adults, VEGFR-3 is expressed in a subset of capillary endothelia, although it is absent in endothelia of all large blood vessels (165). Although lymphatic vessels develop from large veins in the embryonic jugular, retroperitoneal, and perimesonephric regions, only a few adult tissues retain VEGFR-3 expression in the venous endothelia. Such endothelia are seen in the veins of the cartilage channels, vertebral bodies, venous canals of the adrenal medulla, and the splenic venous sinuses (165). Differences in VEGFR-3 expression are also seen between continuous and discontinuous endothelia, the former being negative and the latter positive for VEGFR-3, suggesting that this receptor plays a role in the endothelial transport functions in fenestrated capillaries. VEGFR-2 is also present in the fenestrated endothelia, and it is possible that VEGF-C and VEGF-D secreted by the neuroendocrine cells signal via both receptors in this specific subtype of capillaries in endocrine organs. VEGFR-3 is seen also in endothelia at sites of hematopoiesis or blood cell trafficking, such as in the sinusoids of liver, spleen, and bone marrow, suggesting that it has a regulatory role in the transendothelial translocation of hematopoietic cells (165). Also some nonendothelial expression of VEGFR-3 has been observed in embryonic notochordal cells and in the trophoblasts of the placenta (165, 224).

VEGFR-3 is activated in the blood vessel endothelium in certain pathological conditions, and upregulation of VEGF-C/VEGF-D ligands may accompany this (4, 186, 214). Similarly, VEGFR-2 can be expressed by both blood vascular and lymphatic endothelia (166). During wound healing, acute inflammation is followed by the deposition of fibrin and connective tissue and the growth of blood vessels into the granulation tissue. Most blood vessels then regress as the wound is remodeled into scar tissue.

VEGFR-3-positive lymphatic vessels have been observed to sprout from preexisting lymphatics and grow into the granulation tissue in healing skin wounds (160). These lymphatic vessels persisted in the wound for some time but regressed as the healing proceeded. This suggests that transient lymphangiogenesis is needed during wound healing, in parallel with angiogenesis, and it is possible that inflammatory cells such as macrophages secrete relevant lymphangiogenic factors. On the other hand, no lymphatic vessels were seen in chronic human wounds (160). The absence of lymphatic vessels may contribute to the impaired healing in these conditions. The angiogenic vessels in wound healing remained negative for VEGFR-3, suggesting that there are differences in the regulation of angiogenesis in various pathological conditions.

Little is known about the characteristic features of lymphatic endothelial cells, mainly because isolated lymphatic endothelial cells have not been available for molecular studies. Recently, primary cultures of human dermal endothelial cells were shown to consist of distinct lines of blood vascular and lymphatic endothelial cells (Fig. 4) (121, 144). Cells of the lymphatic lineage could be isolated by antibodies against VEGFR-3 or podoplanin, another marker for lymphatic endothelium. Signaling via VEGFR-3 was shown to be critical for growth, migration, and survival of the isolated lymphatic endothelial cells (144). Also, VEGFR-2 was detected in the lymphatic endothelial cells, suggesting that activation of both VEGF-C receptors may be required for their maximal survival (144, 166). The isolation and culturing of lymphatic endothelial cells now allow more detailed studies of the molecular properties of these interesting cells.

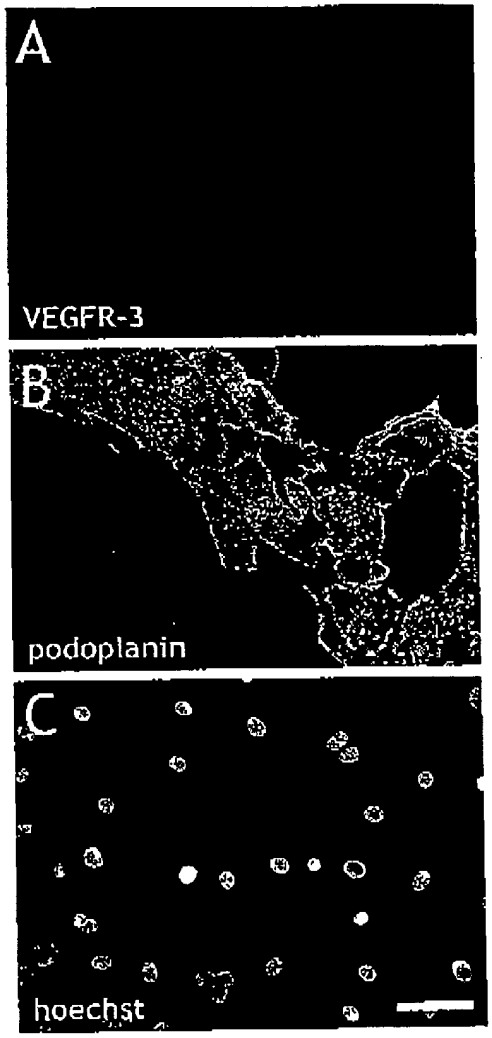

FIG. 4. Cultured microvascular endothelium consists of blood vascular and lymphatic endothelial cells. A subpopulation of cultured human primary microvascular endothelial cells was positive for the lymphatic endothelial cell markers VEGFR-3 (*A*) and podoplanin (*B*). The cells were also stained for nuclear DNA using the Hoechst33258 fluorochrome (*C*). The specific lymphatic cell surface antigens allow the isolation and culturing of stable lineages of lymphatic and blood vascular endothelial cell populations and further studies of the molecular properties of these cells (144). Scale bar in *C* is for *A–C*, 160 μm.

G. Neuropilins Modulate VEGF Activities

NRP-1 and NRP-2 are transmembrane receptor proteins that are required for axon guidance and, according to recent discoveries, also for the regulation of angiogenesis (83, 198, 199). Both neuropilins bind certain isoforms of VEGF, VEGF-E, PlGF-2, and VEGF-B (143, 149, 199, 225). NRP-1 is expressed in the tips of actively growing axons of particular classes of neurons (74), but also in the blood vascular endothelial cells and in certain tumor cells (198, 199). NRP-1 enhances $VEGF_{165}$ binding to VEGFR-2 and VEGF-mediated chemotaxis. Embryos lacking functional NRP-1 die due to defects in VEGF-mediated angiogenesis and subsequent cardiovascular failure (113), and ectopic overexpression of NRP-1 leads to an excess of dilated blood vessels and hemorrhage, apparently due to inappropriate VEGF activity (119). Recently, NRP-2 has been shown to bind VEGF-C and to be expressed together with VEGFR-3 in the endothelial cells of a subpopulation of the lymphatic vessels (106).

H. Angiopoietins and Tie-2 Are Involved in Vessel Stabilization and Maintenance

Tie-1 and Tie-2 (Tek) are expressed in endothelial cells throughout embryonic development as well as in hematopoietic progenitor cells (50). Gene targeting experiments indicate that Tie-1 and Tie-2 are essential to the angiogenic expansion of the vasculature during development. In mouse embryos lacking the Tie-2 receptor, endothelial cells are present in slightly reduced numbers

and are assembled into tubes, but the vessels remain immature, lacking branching networks and proper organization into a hierarchy of large and small vessels (51, 187). The vessels lack intimate encapsulation by periendothelial support cells, and the endocardium is only loosely attached to the myocardium. Thus Tie-2 appears to control the capability of endothelial cells to recruit stromal cells, which stabilize the vessel structure and modulate the function of blood vessels (87). Tie-1 is required cell autonomously for endothelial cell survival and extension of the vascular network during the later part of embryogenesis (173, 187). Vasculogenesis proceeds normally in embryos lacking both Tie-1 and Tie-2, since the angioblasts differentiate normally (172). It appears that one of the earliest critical functions of these receptors concerns endocardial development but that rescue of the embryos is possible if one bypasses the critical period using transgenic expression of Tie-2 (D. Dumont, personal communication).

Angiopoietin (Ang)-1 and Ang-2 bind to Tie-2 with similar affinities, but only Ang-1 can activate the receptor directly. Ang-2 is capable of inhibiting the effects of Ang-1 in endothelial cells in short-term experiments. However, if endothelial cells of human umbilical vein are stimulated with Ang-2 for longer periods, activation of the Tie-2 receptor is obtained (206). Ang-2 is also capable of stimulating Tie-2 in transfected nonendothelial cells. Thus Ang-2 has both agonistic and antagonistic properties, which may relate to its ability to dimerize or oligomerize less efficiently than Ang-1, or to binding to an inhibitor that needs to be downregulated.

Ang-1 is widely expressed in both embryonic and adult tissues (205). Ang-2 is also expressed in embryos around large vessels, but in adults the expression pattern is restricted to sites of physiological angiogenesis, where vascular remodeling occurs (141). Transgenic overexpression of Ang-2 under the Tie-2 promoter in the embryonic endothelium indicates that Ang-2 inhibits the recruitment of supporting perivascular cells, resulting in a phenotype similar to that of the Ang-1 knockout embryos (205). In adults, Ang-2 allows vascular remodeling, which otherwise is restricted by encapsulation by the basement membrane and periendothelial support cells. When the expression of Ang-1 overcomes that of Ang-2, such remodeling ceases and vessels stabilize (reviewed in Ref. 75).

Several lines of evidence indicate that there is significant collaboration between VEGF, Ang-2, and Ang-1 in angiogenic processes. Vascular regression is associated with very high levels of Ang-2 in the absence of activating survival signals from VEGF. In the skin of transgenic mice, VEGF increases the number of capillaries, whereas Ang-1 causes a massive enlargement of postcapillary venules (208). Interestingly, Ang-1 was able to prevent the permeability effects of VEGF (207). Coexpression of both factors was required to obtain an increased number of large vessels in transgenic mice. Surprisingly, a combinatorial function of Ang-1 and Tie-1 was critical for the development of the right-side venous system, but not for the left-side venous system (135). Identification of the ligand(s) for the Tie-1 receptor should provide further insights into the mechanistic basis for this asymmetric regulation of vascular development. Ang-2 may also be involved in the regulation of lymphatic vessels, since knock-out mice lacking functional Ang-2 have chylous ascites with a disorganized and leaky lymphatic vasculature (G. Thurston and G. Yancopoulos, personal communication).

I. PDGFs Are Involved in Recruitment of Perivascular Structures

The PDGF family consists of homodimers or heterodimers made from the pairwise assembly of the related PDGF polypeptide chains (reviewed in Ref. 92). These effects of the PDGFs depend on the target cell type, in particular on the cell's repertoire of PDGF receptors. The dimeric, active receptors consist of α- and β-subtypes of the PDGF tyrosine kinase receptor. The α-receptor can bind PDGF-A, PDGF-B, and PDGF-C chains, whereas the β-receptor is selective for the PDGF-B and PDGF-D chains (21, 23, 132). On the basis of the gene-deficient studies, both receptors are essential for embryonic development. PDGF-A and PDGFR-α are prominently expressed at sites of epithelium-mesenchyme interaction, whereas PDGF-B takes part in blood vessel development (94, 133, 159). PDGF-D is the first known PDGFR-β-specific ligand, and its unique receptor specificity indicates that it may be important in the development and pathophysiology of several organs. The expression of PDGF-C and PDGF-D in the arterial wall and cultured vascular cells suggests that they can transduce proliferation/migration signals to pericytes and smooth muscle cells (213). The expression patterns of PDGF-A and PDGF-C are compatible with overlapping functions, but the situation with PDGF-B and PDGF-D is not that well characterized yet.

The association of supporting smooth muscle cells or pericytes with angiogenic vessels has been suggested to regulate endothelial proliferation, survival, sprouting, and differentiation. During blood vessel development, PDGF-B is expressed in endothelial cells, while pericytes and smooth muscle cells covering the blood vessels express PDGFR-β, indicating paracrine signaling between these two cell types (94, 133). Targeted gene deletion studies of PDGF-B or PDGFR-β gave similar phenotypes. In both mouse strains, blood vessel development was deficient because of the inability of blood vessels to attract pericytes (93, 94, 133). Also the development of the renal and hematopoietic systems was affected (131, 200). Lack of pericytes in embryonic angiogenesis led to hyperplasia of

endothelial cells, supporting the notion that pericytes inhibit endothelial cell proliferation (93).

Smooth muscle cell proliferation in response to the release of growth factors from neighboring cells is one mechanism postulated to account for the development of atherosclerotic lesions. PDGFs may be involved in initiation and progression of atherosclerotic changes in arterial intima by promoting proliferation of smooth muscle cells of the vascular wall (24, 150). These molecules may also have an important role in tumor biology, since expression of the mRNA of the receptors and ligands A and B has been observed in a wide variety of human tumors (92).

J. Ephrins and Ephs Are Involved in Arteriovenous Differentiation

Before the heart starts to beat and circulate blood, the vascular hierarchy must be organized and arteries and veins must be ready to properly transport blood. In studies of the ephrin family of molecules it has become obvious that the fate of the endothelial cell is already marked in early embryonic development when the whole endothelium is still rather uniform in nature (reviewed in Ref. 230). Unlike ligands for other receptor tyrosine kinases, the ephrins cannot act as soluble mediators but are membrane bound to activate their receptors. The Eph receptor family consists of at least 14 members. The receptors, and their ligands, can be divided into two subclasses, A and B (230). Ephrin-B ligands are transmembrane proteins and bind to receptors of the Eph-B subclass. Ephrin-B2 was shown to mark future arteries while its receptor Eph-B4 reciprocally marks the venous endothelium (218). Furthermore, embryos lacking Ephrin-B2 displayed severe defects in vascular remodeling in both arterial and venous domains. These findings provide some of the earliest known markers distinguishing the arterial and venous endothelia. These data for the first time show the existence of bidirectional signaling between these vessel types. This suggests that the molecular differences are at least in part programmed genetically in arterial versus venous endothelia and that these differences may be critical to normal development of the vasculature.

As the ephrins and Eph receptors distinguish arterial versus venous endothelial cells in the primary vascular plexus and are membrane bound, they interact with each other at sites of cell-to-cell contact, and the interactions are considered to lead to cell retraction. This signaling may happen at the junction of arterial and venous cells, or when the developing tubules pass each other and come in contact with adjacent sprouts during early remodeling of embryonic vasculature (230). These local interactions may ensure that new angiogenic sprouts fuse only with their counterpart branches. Inhibitory interactions may also help fusion to occur between the right type of vessels during remodeling, e.g., preventing fusion of arterial and venous structures and ensuring remodeling into a proper capillary network. On the other hand, coexpression of ligands and receptors in the same vessels may provide stimulatory signals to endothelial cells and promote sprouting and morphogenesis resulting in formation of functional vessels (5).

Other members of the Eph family are also critical for the remodeling of the vascular network (5). It has been also shown that the interactions of ephrins and Ephs are not restricted to the arterial/venous boundary but occur throughout vasculature and in mesenchymal cells adjacent to the blood vessels. These endothelial-mesenchymal contact zones may be critical for the patterning of the vasculature.

III. MARKERS OF THE LYMPHATIC ENDOTHELIUM

A major advance in the field of lymphangiogenesis has come from the discovery of lymphatic endothelium-specific markers (Table 1). VEGFR-3 was the first mole-

TABLE 1. *Markers for the lymphatic vessels*

Molecule	Protein Class	Biological Effect	Reference No.
VEGFR-3	Receptor tyrosine kinase on endothelial cell	Lymphangiogenesis survival of LEC	105
LYVE-1	Receptor for extracellular matrix glycosaminoglycan	Transport of HA from tissues to lymph nodes	13
PROX-1	Transcription factor	Developmental lymphangiogenesis	222
Podoplanin	Integral membrane protein	Unknown	30
β-Chemokine receptor D6	Chemokine receptor in afferent lymphatics	Leukocyte recirculation	151
Desmoplakin	Component of intercellular adhering junction in LECs	Adhesion of LECs	168
Macrophage mannose receptor	Receptor in macrophages	Phagocytosis of microbes, viral endocytosis	134

LEC, lymphatic endothelial cell; HA, hyaluronan.

cule found to be expressed in the lymphatic endothelium, but further studies have revealed that it is also expressed in a subset of blood vessels and in addition can be reactivated in the angiogenic vessels in certain pathological conditions (165, 214). Podoplanin is a glomerular podocyte membrane mucoprotein, which occurs together with VEGFR-3 in the lymphatic endothelium and in benign vascular tumors and angiosarcomas, but is also expressed in certain nonendothelial cells, such as osteoblastic cells, kidney podocytes, and lung alveolar type I cells (29, 30). Podoplanin is present in small lymphatics but not in the larger ones having smooth muscle cells, and all the blood vessels as well as high endothelial venules in the lymph nodes are negative for podoplanin expression. Prox-1 is a homeobox transcription factor gene product involved in the growth and elongation of the lymphatic vessel sprouts during development (222). Consistent with this, Prox +/− heterozygous newborns develop chylous ascites and die shortly after birth (222). Prox-1 is also expressed in nonendothelial cells in the lens, heart, liver, pancreas, and nervous system. The third marker, lymphatic vessel endothelial HA receptor-1 (LYVE-1), is a receptor for extracellular matrix/lymphatic fluid glycosaminoglycan in lymphatic endothelial cells (13). This molecule, which is related to the CD44 receptor for hyaluronan, is distributed equally among the luminal and abluminal surfaces of lymphatic vessels and is involved in the uptake of hyaluronan by lymphatic endothelial cells and its transport from the tissues to the lymph (171). However, LYVE-1 is not completely specific for the lymphatic endothelial cells, as it is also present, e.g., in normal hepatic blood sinusoidal endothelial cells (43).

Recently, a β-chemokine receptor D6 was shown to be present in a subset of lymphatic vessels in the skin, intestine, and lymphoid tissues (151). Interestingly, lymphatic vessels in most of the organs remained negative for D6 immunoreactivity. The existence of this receptor on only a subset of lymphatics suggests a functional heterogeneity within the lymphatic vasculature. Consistent with this, recent findings revealed the coexpression of NRP-2 and VEGFR-3 in the lymphatic vessels of the intestine, whereas dermal lymphatic vessels did not show NRP-2 expression (106). The mannose receptor of macrophages is also expressed by lymphatic endothelium in addition to macrophages and other nonendothelial cells (134). The biological role of this receptor in lymphatic vessels is not known, but it may play a role in inflammation and immunity. 5′-Nucleotidase and desmoplakin have also been used to distinguish the lymphatic from the blood vascular endothelium (188, 212), and because lymphatic capillaries lack a continuous basement membrane, immunohistochemistry for extracellular matrix components type IV collagen and laminin have been used to distinguish them from blood capillaries (16). VEGFR-2 is occasionally expressed in lymphatic endothelia (166), and Tie-1 and Tie-2 may also have a role in lymphatic vessel regulation, as they also appear in lymphatic endothelia (103, 166) and mice deficient for the Ang-2 have a lymphatic phenotype (G. Thurston and G. Yancopoulos, unpublished data). In cultured lymphatic endothelial cells, the VEGFR-3, LYVE-1, and podoplanin were essentially located in the same cells (144). In addition, VEGFR-1, VEGFR-2, and VEGFR-3 were expressed in the same cultured lymphatic endothelial cells.

The markers of lymphatic endothelium show overlapping expression patterns in the lymphatic vessels of most benign tissues. However, recent findings have revealed that at least VEGFR-3 and LYVE-1 can be expressed in blood vascular endothelia in physiological conditions (43, 165). More information needs to be obtained to clarify the specificity of these markers in the lymphatic vessels in disease processes. Until then, several markers should be used to confirm the staining of lymphatic vessels in pathological conditions.

IV. DISEASES OF THE LYMPHATIC VESSELS

Pathological processes similar to those that affect blood vessels, such as thrombosis, inflammation, vessel wall hypertrophy, and sclerosis, may also occur to some extent in lymphatic vessels. However, the slow flow of the lymphatic fluid makes lymphatic disorders less acute in character (227). Lymphatic vessel defects are associated with intense fibrosis and overgrowth rather than the dramatic occlusive events such as occur in blood vessels when the blood flow is interrupted.

A. Lymphedema, a Failure of Lymph Transport

An important function of the lymphatic vessels is to regulate the pressure of interstitial fluid in tissues by transporting excess fluid back into the circulation. Edema represents an imbalance between lymph formation and its absorption into the lymphatic vessels. Clinical situations in which the lymphatic system is involved include lymphedema due to impaired lymphatic drainage. This can be caused by inflammatory or neoplastic obstruction of the lymphatic vessels including accumulation of ascites fluid due to lymphatic obstruction in peritoneal carcinomatosis or edema of the arm after surgery or radiotherapy for breast cancer. Lymphatic filariasis, globally the second leading cause of permanent and long-term disability, is a parasitic infection in the lymphatic vessels, which leads to abnormal transport function, massive edema, and deformation of the limbs (227).

Primary lymphedema is a rare developmental disorder in which the transport failure of the cutaneus lymphatic vessels results in interstitial lymph fluid accumulation. Chronic lymphatic dysfunction gradually results in

thickening of the skin, accumulation of adipose tissue, and dermal fibrosis of the affected area (129). Recently, several groups have reported linkage of congenital lymphedema (Milroy's disease) to the VEGFR-3 gene (59, 66, 226) with autosomal dominant inheritance. This mutation was shown to lead to reduced VEGFR-3 tyrosine kinase activity and subsequent failure in transducing sufficient physiological VEGF-C/VEGF-D signals to the lymphatic endothelial cells (107). In all lymphedema families studied, the affected individuals had only one mutant allele (98, 107). This is compatible with the results that inactivation of both VEGFR-3 alleles in mice is embryonic lethal (52).

The mutation affecting the biological activity of VEGFR-3 is probably one cause of primary lymphedema, but some other lymphedema genes also exist, for example, FOXC2 (60). Identification of genetic markers and high-risk members of lymphedema families would facilitate the identification and management of environmental factors influencing the expression and severity of lymphedema. In particular, these findings permit better-informed genetic counseling in affected families and effective therapeutic regimens for lymphedema.

B. Kaposi's Sarcoma Originates From Lymphatic Endothelia

Kaposi's sarcoma is a multicentric neoplasm consisting of multiple vascular nodules appearing in the skin, mucous membranes, and viscera. Typically, the tumor respects tissue planes and is rarely invasive. Molecular and epidemiological studies indicate that development of Kaposi's sarcoma is associated with infection by the human herpesvirus-8 (HHV-8) (204). However, predominantly individuals with specific conditions of immunodysregulation, especially acquired immunodeficiency syndrome patients, develop Kaposi's sarcoma. The nodules are characterized by clusters of spindle-shaped tumor cells and by prominent vasculature consisting of small, irregular, endothelial-lined spaces. It is thought not to be a neoplastic transformation of cells in the classic sense, but rather a manifestation of excessive proliferation of the spindle cells. A central question in the pathogenesis of Kaposi's sarcoma has long been, which cell type in early lesions gives rise to the uniform tumor cells of late nodules (77). The spindle cells are most likely endothelial in origin, but there has been controversy as to whether they are of lymphatic or blood vascular derivation. The pathobiology of Kaposi's sarcoma is complex, and several vascular growth factors have been reported to be expressed in the nodules, suggesting that the balance of angiogenic/lymphangiogenic molecules is behind this tumor type (77).

Several reports support the idea that the cells in Kaposi's sarcoma are of lymphatic endothelial origin. The spindle cells and cells lining the irregular vascular spaces are positive for both VEGFR-3 and podoplanin (30, 103). In addition, the spindle cells have been shown to express VEGFR-2, Ang-2, Tie-1, and Tie-2 (31, 164, 195).

C. Vascular Tumors Express VEGFR-3 in the Endothelium

Vascular malformations are structural anomalies of the vascular system and may be composed of arteries, capillaries, veins, lymphatics, or combinations of them (78). They are often congenital benign lesions present at birth, but their enlargement ceases with the growth of the patient. An activating mutation in the Tie-2 receptor has been shown to cause inherited venous malformations (217). Altered Tie-2 regulation leads to abnormal venous growth or remodeling due to local uncoupling between proliferation and differentiation of endothelial and smooth muscle cells. This resembles the defects in the transgenic mice overexpressing Ang-1 (207, 208). An activating mutation of Tie-2 or overexpression of its activating ligand are both apparently able to cause similar changes in vascular remodeling (reviewed in Ref. 69).

Vascular tumors consist of a broad morphological spectrum from hamartomas to malignant neoplasia. They can be divided into benign tumors (hemangioma) and malignant vascular tumors (angiosarcoma) and to the tumors of lymphatic vessels (lymphangioma) and perivascular cells (glomus tumors). The molecular characteristics of these tumors are so far mostly unknown, but VEGF and its receptors have been shown to be expressed in the endothelial cells of these tumors (180). Although normal mesenchymal tissues show VEGFR-3 in the lymphatic endothelial cells, cells of benign and malignant vascular tumors show widespread VEGFR-3 expression, suggesting that VEGFR-3 is upregulated in the proliferating blood vascular endothelial cells. This is consistent with the VEGFR-3 expression in the embryonic developing vessels, as well as reactivation in adult angiogenic blood vessels (52, 214). Although strong expression of VEGFR-3 would be consistent with lymphatic differentiation, the extensive erythrocyte content in the vascular lumina of these lesions supports the idea that VEGFR-3 expression in these tumors reflects a proliferative vascular phenotype rather than a lymphatic phenotype. The expression of VEGFR-3 among vascular proliferations demonstrates that blood vessel endothelia can acquire VEGFR-3 expression independently of lymphatic vascular differentiation.

Classification of angiosarcomas is mostly based on morphological criteria. It has been suggested that some of the angiosarcomas contain components of a lymphatic lineage, but since the antibodies used for the detection of blood endothelia show overlapping staining of lymphatic

endothelia, there is no proof of this. Some cell populations in angiosarcomas are positive for podoplanin, which retains its lymphatic endothelial cell specificity in vascular tumors, supporting the idea of mixed expression of both blood and lymphatic phenotypes in angiosarcomas (30). As expression of VEGFR-3 is seen in the majority of benign and malignant vascular tumors (164), it could thus be used as a lineage marker to identify endothelial cell differentiation in the tumors. However, further studies are needed to evaluate the sensitivity and specificity of both VEGFR-3 and podoplanin in the malignant transformation of blood or lymphatic endothelial cells.

Lymphangiomas result from abnormal development of lymphatic vessels, which prevents lymph fluid draining from the affected area. Lymphangiomas can originate in most organs, although they are most often found in the soft tissues of the head and neck (cystic hygroma) and axilla. They consist of a benign multicystic mass of dilated networks of lymphatic channels. Both VEGFR-3 and podoplanin specifically stain the endothelia of lymphangiomas and could be used for diagnostic purposes (30, 103, 164).

V. TUMORIGENESIS AND METASTASIS

Cancer is a disease involving dynamic changes to the genome. Mutations produce oncogenes that gain a dominant function, and tumor suppressor genes become inactivated and lose function (reviewed in Ref. 88). Lots of evidence indicates that tumorigenesis in humans is a multistep process, and these steps reflect the genetic alterations that drive the progressive transformation. Cancer cells have defects in regulatory circuits that govern normal proliferation and homeostasis. Whereas normal cells require mitogenic signals before they can move from a quiescent state into an active proliferative stage, malignant cells are self-sufficient for the growth signals and insensitive to the growth-inhibitory signals, which normally operate to maintain cellular quiescence and tissue homeostasis. Tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulation from the normal tissue microenvironment. They develop various strategies to avoid terminal differentiation and are therefore more capable of proliferating than the well-differentiated benign cells. Most soluble mitogenic growth factors are produced by one cell type to stimulate the proliferation of another, whereas many cancer cells acquire the ability to synthesize their own growth factors leading to autocrine stimulation. It has long been thought that tumors are independent from the surrounding cells and their action, but now it seems more likely that cancer development depends on interactions between tumor cells and their benign neighbors. Tumors and metastases tend to harbor complex mixtures of several cell types that collaborate to create a malignant growth, including fibroblasts, immune cells, and endothelial cells (68, 88).

The cells within aberrant proliferative lesions initially lack angiogenic ability, preventing their expansion. The ability to induce and sustain angiogenesis seems to be acquired in discrete steps during tumor development via an angiogenic switch from vascular quiescence to proliferation (88). In studies of transgenic mouse tumorigenesis (Rip-Tag), angiogenesis was found to be activated in mid-stage lesions before the appearance of full-blown tumors (70). These observations indicate that neovascularization is necessary for the rapid clonal expansion associated with the formation of macroscopic tumors. Tumors appear to activate the angiogenic switch by changing the balance of angiogenic inducers and inhibitors (20, 68). Another regulatory mechanism is the function of proteases, which can control the availability of angiogenic activators and inhibitors stored in the extracellular matrix (48). There is also evidence of heterogeneity of angiogenic activity in malignant neoplasms, where this seems to be regulated by the organ microenvironment (194).

Tissue hypoxia is a fundamental angiogenic stimulus characteristic also of malignant tumors. The VEGF gene has been shown to contain specific hypoxia-responsive elements and to be upregulated in response to low oxygen tension (65). Ang-2 levels are also increased by hypoxia, suggesting a collaboration of VEGF and Ang-2 in the regulation of neovascularization of ischemic tissue (reviewed in Ref. 126). Tumor-derived signals such as VEGF may specifically induce Ang-2 expression in tumor endothelia, and this may be one important component in angiogenic switch and in the formation of an endogenous tumor microcirculation.

A. Lymphatic Vasculature and Growth Factors in Tumors

As tumors need neovascularization to grow, microvascular density has been used as a measure of tumor angiogenesis, and its correlations to tumor growth, metastasis, and prognosis have been studied (221). Levels of VEGF are upregulated in a large number of human tumors (for review, see Ref. 65), and inhibition of VEGF activity results in the suppression of growth of a wide variety of tumor cell lines in murine models (63). It was long thought that lymphatic vessels may be lost, collapsed, or could not penetrate in the expanding primary tumors because they cannot survive in the high solid stress inside the tumors (reviewed in Ref. 167). However, recently some intratumoral lymphatic vessels have been observed (201), and an interesting question now is whether or not lymphatic vessel density, compared with blood vascular density, is related to prognosis and metastatic spread.

Few data are available on the influence of lymphatic microvessel density on survival in cancer, because until recently there was no reliable immunohistological marker for the lymphatic endothelium. In ovarian cancer, the lymphatic vessel density had no influence on the progression of the disease, and in cervical cancer an increased amount of lymphatic vessels may even be associated with a favorable prognosis (25, 26). It is likely that human tumors demonstrate heterogeneity with regard to the presence or absence of intratumoral lymphatics. The nature of the marker may also influence the determination of the lymphatic vessel density. VEGFR-3 has been seen in the endothelial cells of the proliferating vasculature in solid tumors and vascular tumor cells of endothelial origin (164, 214) and therefore cannot be used alone to confirm the intratumoral lymphatic vessel density. Also LYVE-1 has been shown to be expressed by the sinusoidal blood vessels of the liver (43). Therefore, until the specificity of these markers in lymphatic vessels during tumor progression and metastases has been clarified, multiple markers should be used in elucidating correlation between lymphatic vessel density and tumor growth, metastases, and prognosis.

All VEGFRs are present in tumor neovasculature, and tumor cells have been reported to be able to secrete VEGF, VEGF-B, VEGF-C, and VEGF-D (4, 65, 186). However, the angiogenic switch is thought to be carefully regulated, and at least some specific genetic events in tumor progression correlate with lymphatic metastasis, suggesting that a "lymphangiogenic switch" mechanism is also a formal possibility.

B. Mechanisms of Blood Vascular and Lymphatic Metastasis

The capacity to spread enables cancer cells to escape the primary tumor mass and colonize new areas in the body, where nutrients and space are not limiting. Tumor cell dissemination is mediated by mechanisms including local tissue invasion, lymphatic spread, hematogenous spread or direct seeding of body cavities or surfaces (47). Although the biochemical mechanisms are not completely understood, it is thought that the metastatic spread of a tumor is not a random process. Distinct patterns of metastasis can be discerned that vary from tumor type to tumor type. A common pattern for carcinomas is that regional lymph nodes are often the first sites to develop metastases either draining via preexisting afferent lymphatic vessels and/or via newly formed lymphatic capillaries. This pattern of metastasis is central to the utility of the sentinel lymphnodectomy as a surgical technique. However, not all tumors and tumor types metastasize to the regional lymph nodes first. The mechanisms determining whether regional lymph nodes or other sites first develop metastases remain poorly understood. In fact, most disseminated tumor cells have a limited life span, and only a few develop into clinically detectable micrometastases. Nevertheless, identification of those occult tumors cells, and prevention of their growth and spread would be of great clinical significance.

The presence of a metastasis in a lymph node does not necessary mean that the tumor cells have been arrived via the lymphatic vessels (215). Intra-lymphatic tumor cells may pass directly into the blood vascular system through venolymphatic communications; that has been observed in certain organs. Also, an increase in the number of tumor cells in the blood circulation should also raise the frequency of the lymph node metastases. This pathway may be related to the increased number of microvessels in the tumors and therefore cannot be considered as lymphatic vessel-mediated metastasis.

C. VEGF-C, VEGF-D, and Tumor Metastases

VEGFR-3 may play an important role in the formation of tumor-induced neovascularization, since it is expressed in capillary vessels during tumor angiogenesis (164, 214). Inactivation of VEGFR-3 by neutralizing antibodies suppressed tumor growth by destabilizing large vessels in tumor xenografts in mice. Microhemorrages were seen in these vessels, suggesting that VEGFR-3 could be involved in maintaining the integrity of the endothelial cell lining in the neovasculature (122). Frequent VEGFR-3 antibody administration was required for the suppression of tumor growth, but the architecture of the nonangiogenic blood and lymphatic vessels remained unaffected. It has also been shown that even a prolonged suppression of VEGF activity in adult mice has no effect on the maintenance of the vascular system, although it suppressed angiogenesis severely in embryos (81). The fully established blood and lymphatic vessels seem to be resistant to treatment with these kinds of antiangiogenic agents.

Recent work using experimental models has highlighted the role of VEGF-C and VEGF-D in tumor biology. Transgenic mice overexpressing VEGF-C in β-cells of the endocrine pancreas (Rip-VEGF-C, rat insulin promoter) developed extensive lymphangiogenesis around the endocrine islets of Langerhans (145). Furthermore, when tumors were induced in these VEGF-C overexpressing islets, by mating the mice with transgenic mice expressing the simian virus 40 T-antigen oncogene in the β-cells (Rip1-Tag2), metastatic tumor cell aggregates of β-cell origin were observed in the surrounding lymphatic vessels. These mice also frequently developed metastases in the lymph nodes, which drain the pancreas, whereas tumors in mice lacking the VEGF-C transgene never metastasized, nor were tumor cells observed inside the lymphatic vessels (145). VEGF-C overexpressed by the

tumors did not significantly alter tumor volume, transition from adenoma to carcinoma or tumor angiogenesis, but interestingly the tumor incidence was increased, for as yet unknown reasons. Similarly, human breast cancer cells expressing ectopic VEGF-C were shown to induce lymphangiogenesis in and around the orthotopically implanted tumors (Fig. 5*A*) (111, 197). However, VEGF-C did not have a significant effect on angiogenesis, although it increased tumor growth. Increased spreading of the cells to the regional lymph nodes was observed, and the degree of tumor lymphangiogenesis correlated with lymph node metastases (M. M. Mattila, personal communication). VEGF-C-induced tumor growth, lymphangiogenesis, and intralymphatic tumor growth was inhibited by adenoviral expression of the soluble VEGFR-3 receptor (Fig. 5*B*) (111). However, although VEGF-C is present, it is not always sufficient to induce the formation of functional lymphatic vessels (128). There may be variation between different tumor types, and the solid stress exerted by the growing tumor may prevent the growth of lymphangiogenic sprouts into the tumor.

VEGF-D was also shown to promote the metastatic spread of tumor cells via the lymphatics (201). In addition to lymphangiogenesis and increased metastases, the tumors secreting VEGF-D also had an increased growth rate and tumor angiogenesis. The growth of the tumor, angiogenesis, and formation of metastases were inhibited by neutralizing antibodies against the bioactive region of VEGF-D. The differences between the tumor angiogenic properties of VEGF-C and VEGF-D may be due to differences in their proteolytic processing in different tumors. Some of the heterogeneity in the effects of these growth factors may also result from variable expression of their receptors, VEGFR-2 and VEGFR-3, on the blood vascular and lymphatic endothelia. In particular, in the above case, enhanced tumor angiogenesis was probably obtained for VEGF-D because of its increased proteolytic processing, which resulted in an increased affinity to VEGFR-2 (201),

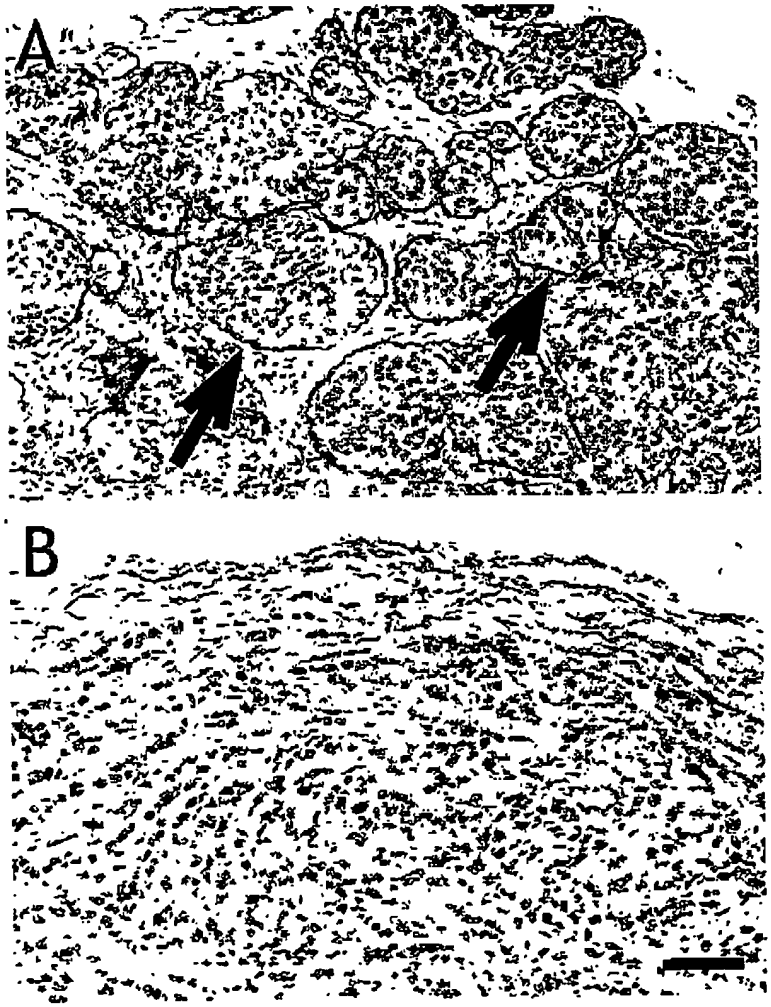

FIG. 5. Development of metastasis via the lymphatic vessels. Overexpression of VEGF-C in xenotransplanted human breast carcinoma cells in mice leads to tumor lymphangiogenesis and accumulation of metastatic tumor cells in newly formed peritumoral lymphatic vessels (stained for the lymphatic marker LYVE1; arrows in *A*). Such lymphangiogenesis and intralymphatic tumor growth was prevented by adenoviral delivery of a soluble VEGFR-3 protein (*B*). Scale bar in *B* is for *A* and *B*, 200 μm.

compared with the VEGF-C models. Consistent with this, the vascular effects of VEGF-C in experimental tumors were reduced, when the mice were treated with blocking antibodies against VEGFR-2 (104). Also, intratumoral lymphatic vessels were observed in tumor xenografts (111, 197, 201), but not in the transgenic tumors (145), which may be at least partially explained by the trapping of vessels in between the rapidly growing tumor foci in the xenografts. On the basis of these observations, tumor vessel formation can be dissected into pathways that preferentially activate angiogenesis (driven by VEGFR-2) and pathways that preferentially activate lymphangiogenesis (driven by VEGFR-3), although there is evidence that the receptors share overlapping expression patterns (166).

Activation of lymphatic endothelial cells by tumor cell secreted factors may promote the interaction of tumor cells with the lymphatic endothelial cells, and thereby facilitate tumor cell entry into the lymphatics. In spite of the increased metastatic tendency of VEGF-C-overexpressing tumor cells, metastases were only seen in about one-third of the tumor-bearing mice. Overall, on the basis of studies in which VEGF-C or VEGF-D has been overexpressed in tumors, one could suggest that there are additional, rate-limiting steps in the metastatic process. The simplest explanation for the metastasis-enhancing effects of VEGF-C and VEGF-D is that they eliminate one rate-limiting step by increasing the surface area between invading tumor cells, which is in contact with the hyperplastic lymphatic endothelium. However, they could also facilitate metastasis by increasing vascular permeability, by changing the adhesive properties or cytokine or chemokine expression patterns of the lymphatic endothelium. VEGF-C and VEGF-D secreted by the tumor cells could also have important effects on the tumor interstitial fluid pressure. Both can increase vascular leak, but not as efficiently as VEGF; a parallel increase in lymphangiogenesis could alleviate this effect. The increased interstitial fluid pressure could be a major determinant of tumor cell seeding into the blood vascular and lymphatic circulation, especially since recent studies have shown that a proportion of the lumen of tumor blood vessels themselves consists of tumor cells (44, 90).

It is still unknown whether VEGF-C or VEGF-D expression also promotes lymphangiogenesis in human tumors, and if so, does this increase the rate of metastasis to the lymph nodes. VEGF-C expression has been detected in about one-half of human cancers analyzed (186). In breast cancer, VEGF-C expression seems to correlate with lymph node-positive tumors, whereas VEGF-D may be expressed predominantly in inflammatory breast carcinomas, suggesting that these growth factors have distinct roles in various tumors despite their biochemical similarities (124). A number of reports have described a correlation between VEGF-C expression in human tumors and the formation of metastases in regional lymph nodes. So far, VEGF-C levels in primary tumors have been shown to correlate significantly with lymph node metastases in thyroid, prostate, gastric, colorectal, lung, and esophageal carcinomas (6, 32, 118, 152, 211, 231). Less is known about the presence of VEGF-D in human tumors, but VEGF-D was shown to be upregulated in human melanomas compared with melanocytes (4). In melanomas, VEGF-D was detected in the tumor cells and in vessels adjacent to immunopositive tumor cells, but not in vessels distant from the tumors. This suggests that VEGF-D binds to the endothelial cells of nearby vessels and contributes in a paracrine manner to the regulation of tumor angiogenesis.

It is not known to what extent tumor cell secreted factors are directly responsible for the large lymphatic vessels occasionally detected around human tumors. Inflammatory cells for example could contribute to the lymphangiogenesis, as VEGF-C is chemotactic for macrophages and readily induced by proinflammatory cytokines (58, 178). Dilated and engorged peritumoral lymphatics may function poorly because of the obstruction of the lymphatic vessel by tumor cells. There is evidence that the lymph flow in peritumoral lymphatics can change to lymphaticovenous communication or reverse lateral flow (229). It may be that lymph retention and reflux of lymph induced by vessel obstruction favors the metastasis to occur by direct seeding to body cavities or by hematogenous spread. It is not clear whether the newly formed lymphatic vessels mature in a similar way to the blood vessels or whether they are more prone to tumor cell invasion, for example, because of differences in the expression of adhesion receptors. VEGF is known to be able to upregulate the expression of adhesion molecules in the vasculature, but such a role for VEGF-C and VEGF-D is not known.

Although it seems evident that both VEGF-C and VEGF-D can induce the growth of new lymphatic vessels, several questions remain unanswered regarding tumor lymphangiogenesis and metastasis. For example, it is not known whether it is sufficient for preexisting lymphatic vessels to expand by circumferential growth or whether new vessels are required for the enhancement of the metastatic process. On the other hand, lymphatic vessels may either actively penetrate into existing tumors or become trapped between expanding tumor foci. The intratumoral lymphatic vessels observed are usually collapsed due to the high tissue solid stress on solid tumors; this may impair their transport capacity. Also, as in angiogenesis, lymphangiogenesis may occur by several mechanisms, and different regulatory factors may be involved.

VI. IMPLICATIONS FOR THE HUMAN DISEASE THERAPY

A. Antiangiogenic and Antimetastatic Therapy

Despite advances in surgery, radiotherapy, and chemotherapy, the prognosis of many cancers remains poor. One of the goals of gene therapy in cancer treatment is to target the therapeutic gene to all tumor cells, as each untreated tumor cell has the potential to progress and to metastasize. The purpose of combining conventional cancer therapy with antiangiogenic agents is that the antivascular effects of the chemotherapy and radiotherapy are selectively enhanced in the cells of newly formed vessels, for example, when survival signals mediated by VEGF are blocked (61, 115). However, one needs also to consider the unwanted toxic effects of the cancer therapy on the vasculature, some of which could be alleviated by provision of vascular survival factors (162). Therapy resistance in tumor cells depends on tumor cell heterogeneity, genetic instability, and a high mutation rate. Compared with conventional cytostatics, there may well be less of a risk of resistance to antiangiogenic agents, since the endothelial cell compartment is assumed to be genetically more stable and have a lower mutation rate than the tumor cells (27, 61). However, the immature nature of tumor blood vessels should provide a therapeutic window where the tumor vascular endothelium can be targeted leaving the rest of the vasculature intact.

Several antiangiogenic agents, alone or in combination with conventional therapies, have advanced to clinical trials. Many of them target angiogenic growth factors, their receptors, or downstream signaling. For example, neutralizing antibodies against VEGF or VEGFR-2 have been used in the treatment of various solid tumors with and without combination with traditional cancer therapy (39). Although preclinical results are promising, it is not yet clear how antiangiogenic therapies will perform clinically. As mechanisms of angiogenesis differ in various tissues, therapeutic inhibition of angiogenesis needs to be modified for each target tissue (63). There is evidence indicating that different types of tumors have distinct molecular mechanisms to activate the angiogenic switch. Whether a single antiangiogenic molecule will suffice to treat all tumor types, or whether an ensemble of such molecules needs to be developed, remains to be seen. Toxic or vaso-occlusive therapy has also been used to target directly tumor vasculature (8, 56, 84).

The differences between the surface molecules of blood vascular and lymphatic endothelia can be taken into account when targeting therapeutic agents selectively to tumor lymphatic vessels. This would increase the potency of the drug in the target tissue and limit the possibility of side effects (9, 181). Methods such as cDNA microarray analysis and phage display screening have been used to identify such markers. The targeting of lymphatic vessels in human tumors would help in imaging these vessels and facilitate studies into the role of lymphatic vessels in the metastatic processes. Anticancer drugs specifically targeted to peritumoral lymphatic vessels could be used to inhibit lymphatic metastasis. However, the destruction of these vessels would further elevate the high interstitial fluid pressure inside the tumors, impairing the delivery of other drugs. Because VEGF-D expression has been shown to become upregulated by direct cell-cell contacts, the increased intratumoral pressure could increase close contacts between the tumor cells and lead to a compensatory increase of the lymphangiogenic growth factor levels (158). Increased intratumoral fluid pressure could also enhance the likelihood of hematogenous metastasis (39, 203).

B. Gene and Recombinant Protein Therapy of Myocardial and Peripheral Ischemia

Ischemic heart disease stems from poor oxygenation of the heart muscle as a consequence of coronary vessel obstruction (47). Promoting angiogenesis in this situation may have a positive impact by increasing collateral vessel formation. Similarly, ischemia in the lower limb would also be alleviated from such an improvement. Animal studies on proangiogenic therapies on these conditions have shown promising results (63). However, studies of Buschmann and Schaper (33) have shown that collateral formation is critically dependent on arteriogenesis, which is a distinct process from angiogenesis.

Various angiogenic approaches to treating ischemic diseases are already in clinical trials (63, 99). Many of them involve the delivery of VEGF to ischemic tissue to stimulate the growth of new vessels. One outstanding question is whether a single angiogenic factor can promote functional and sustainable angiogenesis, or if a combination of angiogenic molecules is required. For example, vessels induced by VEGF are leaky and tortuous, so it may be possible to control leakiness by combining VEGF with Ang-1, as was done in a mouse model (207). Recombinant VEGF-C may also be used as a therapeutic angiogenic growth factor in the treatment of tissue ischemia, possibly even in combination with VEGF (95). The angiogenic activity of VEGF-C in ischemic conditions may relate to the increased expression of VEGFR-2 and the presence of relatively high endogenous VEGF levels in such conditions. On the other hand, lymphangiogenesis has never been studied in ischemia, but no evidence exists at present concerning the possible role of hypoxia in the regulation of the lymphatic vessels. The findings that VEGF has an important role in bone angiogenesis and endochondral bone formation suggest that these factors

could also be used to enhance revascularization in orthopedic conditions such as nonhealing fractures (80).

An important question concerning the proangiogenic therapies is how the therapeutic molecules should be administered. Is it possible to deliver a potent molecule like VEGF in therapeutic quantities without causing toxic side effects, like hypotension or edema? Suitable methods and routes of therapy would also avoid the infiltration of inflammatory cells, such as macrophages, which express VEGFR-1. It is not clear for how long these factors should be administered, whether the therapy leads to a functional vasculature and whether the vessels will regress upon the completion of therapy. On the other hand, the studies of Dvorak and co-workers (170) clearly show that at least some of the vessels generated in response to VEGF gene therapy eventually stabilize and acquire periendothelial structures. Such stabilization of vessels may depend on the level of intraluminal blood flow. However, concern about potential side effects, such as inappropriate blood vessel growth in patients with diabetic retinopathy or solid tumors, has decreased the enthusiasm for the use of these powerful agents (220).

New endothelial cells must be recruited in the beginning of angiogenesis. This recruitment is from two possible sources, preexisting vessel endothelia and endothelial precursor cells from the blood circulation (174). The existence of angioblast-like circulating endothelial precursor cells in adults has only recently been suggested, and their role in supporting postnatal angiogenesis is under intensive investigation (174). VEGFR-2 has been suggested as a marker for these cells. The ex vivo expansion of these precursors would be useful for the promotion of vascular healing, for provision of suitable cellular coatings for vascular grafts, or for the delivery of toxins to the tumor vascular bed (174). Mobilization of such precursors from bone marrow could also accelerate healing at sites of vascular trauma.

C. Therapeutic Lymphangiogenesis

At present, lymphedema is treated by manual lymphatic drainage and by compressive garments. The discovery of specific genes involved in the regulation of lymphatic vessels and in the pathology of lymphedema should make the design of more targeted treatments for this disease possible. Because transgenic VEGF-C/D overexpression is able to induce the postnatatal growth of new lymphatic vessels in the skin (100, 216), these molecules may also be useful in lymphedema patients. Subcutaneous adenoviral gene transfer of VEGF-C in mice has already been shown to induce lymphangiogenesis within 2 wk of treatment (57). A mouse model (Chy), which mimics human lymphedema, allows the study of possible gene therapies (106). These mice, like the human patients, have a heterozygous mutation in the VEGFR-3 gene, resulting in partial loss of VEGFR-3 activity (106, 136, 137). This impairs the development of the cutaneous lymphatic vasculature and leads to hypoplastic, nonfunctional vessels. Secondary to this, there is thickening of the skin as well as accumulation of adipose tissue and dermal fibrosis similar to the lymphedema patients.

The effect of VEGF-C was explored by both gene therapy and transgene approaches in the Chy model (106). When VEGF-C was overexpressed in the skin of Chy mice, growth of functional cutaneous lymphatic vessels was induced, suggesting that VEGF-C/D gene therapy may be applicable to human lymphedema. Such therapy could also be used in nonhereditary, regional forms of lymphedema resulting from trauma, surgery, or lymphatic vessel destruction after filariasis. Because VEGFR-3 signaling plays a role in lymphatic endothelial cell survival (142, 144), long-term growth factor expression may be needed to obtain lymphangiogenesis and maintain these vessels in chronic lymphedema. The functional characteristics of the newly formed lymphatic vessels, for example, their connections to draining lymphatic vessels, still require additional studies.

The larger connecting lymphatic vessels, the lymphatic vessels of the visceral organs, as well as the VEGFR-3-positive fenestrated blood vessels appeared normal in the lymphedema mice (106). Consistent with this, skin is the most affected organ also in lymphedema patients. Interestingly, the lymphatic vessels of transgenic mice expressing a soluble, circulating form of VEGFR-3 (which results in reduced VEGF-C/D signaling via their lymphatic endothelial receptor) regress before birth, but start to regrow later during the postnatal period in the internal organs (142). However, in the skin, the lymphatic vasculature remains hypoplastic. These results support the view that the cutaneous lymphatic vessels are regulated differently from those in other organs and that besides VEGFR-3, there are additional signals for growth and maturation of the lymphatic endothelium postnatally. For example, it could be that the extracellular matrix provides signals via the integrins, which activate the VEGFR-3 pathway (219). NRP-2, which binds VEGF-C and is expressed in the lymphatic vessels of internal organs but not in the skin, may also be one such factor (106). It is possible that NRP-2 is involved in VEGFR-3-mediated signal transduction at sites where the two receptors are coexpressed, which is similar to what has been reported for NRP-1 regulation of VEGFR-2-mediated signals (199).

The mechanisms of lymphangiogenesis in adults have not been elucidated. The generation of lymphatic vessels could in principle require endothelial cell sprouting from, or splitting of, preexisting lymphatic or blood vessels; in situ differentiation of endothelial cells; or recruitment and lymphatic differentiation of endothelial precursor cells, as has been described in other models

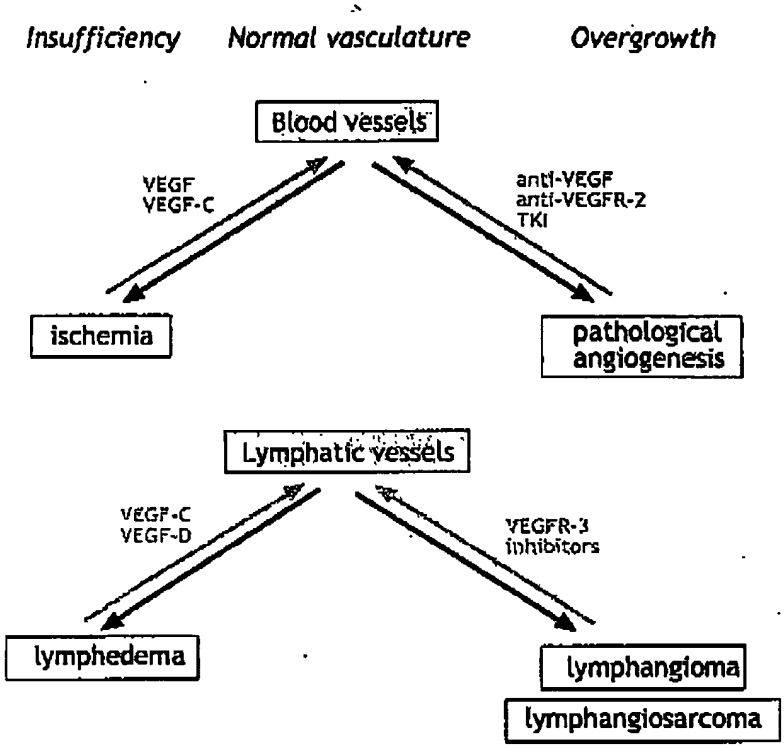

FIG. 6. Pathological conditions of blood and lymphatic vessels and possible therapeutic interventions. *Top*: inefficient blood circulation leads to ischemic tissue (black arrow on *left*). Ischemia could be treated by VEGF- or VEGF-C-induced angiogenesis (gray arrow on *left*). On the other hand, overgrowth of the blood vessels in diseases associated with pathological angiogenesis could be circumvented using antibodies that neutralize the activity of VEGF or by tyrosine kinase inhibitors (TKI) (arrows in *right*). *Bottom*: insufficient signaling to lymphatic endothelial cells via VEGFR-3 leads to lymphedema, the accumulation of the lymph fluid in the tissue. VEGF-C and VEGF-D are both able to induce lymphangiogenesis, and this could be used to treat lymphedema (gray arrow on *left*). Overgrowth of lymphatic endothelia seen in lymphatic vascular tumors (lymphoma and lymphangiosarcoma) could be treated by VEGFR-3 inhibitors.

(10, 189, 192). Results obtained so far are comparable to the processes of sprouting from preexisting vessels and splitting of large dilated lymphatic vessels, which are seen after VEGF-C treatment (57). However, upregulation of VEGFR-2 and VEGFR-3 on endothelial cells was seen in response to VEGF-C, raising the interesting possibility that endothelial cells in blood vessels may also participate in lymphangiogenesis by the process of migration and *trans*-differentation. Furthermore, at least in the avian system, precursor cells or lymphangioblasts have been demonstrated, from which new lymphatic growth may initiate (189).

VII. CONCLUSIONS

The development and regulation of endothelial cells require the orchestration of many growth factors in a carefully coordinated manner. Blood and lymphatic vessels are formed in an interactive manner during embryonic development, but both vessel types are maintained in a rather quiescent state in adults and are active only in sites of new tissue growth. Angiogenesis has been extensively studied for over a decade, but lymphangiogenesis is a relatively new topic for vascular biology. The discovery of specific molecules involved in the biology of the lymphatic vessels now enables a more extensive study of the many roles of the lymphatic vessels. VEGF-C and VEGF-D both stimulate the growth of lymphatic vessels, being the first growth factors found for lymphatic endothelial cells. Similarities between the regulation of blood and lymphatic vessels have been observed, and these two vessel systems seem to work in a tightly regulated manner. Lymphangiogenesis may occur in sites of angiogenesis, either following the growth of blood vessels, as in wound healing, or independently as has been observed in experimental tumor models and in inflamed tissue (D. McDonald, personal communication). The findings made so far on lymphatic regulation will be helpful in the diagnosis of certain vascular tumors and in designing specific treatments for lymphedema. Regulation of the blood and lymphatic vessels and some possible therapeutic approaches have been summarized in Figure 6. Many promising experiments have been done concerning the inhibition of metastatic spread of tumor cells via the lymphatic vasculature. The isolation and culture of lymphatic endothelial cells now offer better tools to study the molecular characteristics of these cells. Encouraging results on the therapeutic use of angiogenic and lymphangiogenic molecules have been obtained in experimental models, and ongoing and future clinical trials will show the therapeutic potential of the molecules of VEGF family of growth factors and receptors.

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Appendix III: Related Proceedings

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